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The Effect Of Individual Milk Proteins On Bioaccessibility Of Green Tea Flavan-3-Ols

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THE EFFECT OF INDIVIDUAL MILK PROTEINS ON BIOACCESSIBILITY OF GREEN TEA FLAVAN-3-OLS

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To my family for their inspiration and support.

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TABLE OF CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

ABBREVIATION	DESCRIPTION
EGC	Epigallocatechin
EC	Epicatechin
EGCG	Epigallocatechin-gallate
ECG	Epicatechin-gallate
GT	Green Tea
JK	Jenness Koops Buffer
HPLC	High Performance Liquid Chromatography
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
$\%F_q$	Percent fluorescence quenched
Trp	Tryptophan

ABSTRACT

Moser, Sydney Elizabeth. M.S., Purdue University, December 2013. The Effect of Individual Milk Proteins on Stability of Green Tea Flavan-3-ols. Major Professor: Mario G. Ferruzzi.

While information regarding the impact of flavan-3-ol-protein interactions on food quality attributes (flavor, texture, and physical stability) exists, insight into the potential consequence of these interactions on bioavailability of health-promoting flavan-3-ols remains unclear. The ability of nonspecific protein interactions in flavan-3-ol rich foods and beverages to alter digestive stability and/or digestive release (bioaccessibility) of flavan-3-ols has been previously proposed but unconfirmed. The primary objective of this study was to characterize effects of individual milk proteins and the milk matrix on *in vitro* bioaccessibility of green tea flavan-3-ols from model beverage systems. As a secondary objective, the impact of protein gastrointestinal digestion on protein-flavan-3-ol binding was assessed using fluorometry. Protein solutions containing sodium-caseinate (S-CSN), α-lactalbumin (α-LA), β-lactoglobulin (β-LG) (0.356 mg/100 mL, 0.01 mg/100 mL, 0.035 mg/100 mL, respectively), or non-fat dry milk (NFDM, 0.1 mg/mL) were prepared in Jenness Koops buffer containing milk salts or dd water and formulated at dosages of 10, 20, and 40% (v/v) into model green tea beverages containing 60 mg/100 mL total flavan-3-ols. Samples were then subjected to *in vitro* digestion simulating

human gastric and small intestinal conditions. Pre- and post-digestion flavan-3-ol profiles were assessed by HPLC and relative (%) and absolute (μmol/g) bioaccessibility of individual flavan-3-ols were calculated. With regards to green tea beverages formulated to 40% with milk protein solutions, inclusion of S-CSN significantly decreased ($p<0.05$) bioaccessibility of all flavan-3-ols, β-LG significantly decreased ($p<0.05$) bioaccessibility of all flavan-3-ols except EGC, and α-LA significantly decreased ($p<0.05$) bioaccessibility of only gallated catechins compared to buffer controls. Furthermore, S-CSN effects on flavan-3-ol absolute bioaccessibility were more notable compared to whey proteins, decreasing bioaccessibility of EGC, EC, EGCG, and ECG from 98.29±9.22, 30.88±0.52, 40.52±1.16, and 6.51±0.16 μmol/g, respectively, from JK buffer control to 17.82±4.68, 21.72±1.23, 11.29±1.18, and 2.86±1.20 μmol/g, respectively. Interestingly, milk minerals from JK buffer increased flavan-3-ol stability during digestion compared to water control, significantly increasing ($p<0.05$) absolute and, by extension, relative bioaccessibility from 18 to 32%.While some individual trends were exhibited, no overall significant dose-dependent effects ($p>0.05$) were observed at protein and JK levels tested. SDS-PAGE results demonstrated only partial digestion of protein after peptic digestion, and degradation of proteins to <10 kDa after only 15 minutes of duodenal digestion. These results compared well with fluorometry results suggesting that maximum binding of S-CSN by EGCG, the main flavan-3-ol in green tea, occurs when protein is partially digested by pepsin in the gastric phase. Further digestion by pancreatin in the small intestinal phase appeared to reduce capacity for binding. Combined these data suggest that interactions between individual milk proteins and flavan-3-ols may alter bioaccessibility. However, the extent of these effects is subtle and

dependent on the ability of individual interactions to survive normal digestive conditions. Interestingly, salts typically found in milk serum may exert protective effects on polyphenols from tea, stabilizing them to digestive reactions that may lead to their degradation.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Both epidemiological and experimental studies support the notion that specific plant derived polyphenols may impact chronic disease risk and health outcomes (Scalbert & Williamson, 2000). While promising, extension of these findings to new food and beverage systems is challenged by the poor bioavailability of polyphenols generally reported from foods. Several factors are believed to limit polyphenol bioavailability including digestive release from food matrices (Mandalari et al. 2013), instability to digestive conditions (Krook et al. 2012; Bermúdez-Soto et al. 2007; Green et al., 2007; Tagliazucchi et al. 2010), poor intestinal absorption, and rapid metabolism and clearance (Chow et al. 2005; Lee et al. 2002). Interactions between polyphenols and specific food components are also believed to alter stability and release of polyphenols and potentially modulate their bioavailability (Peters et al. 2010; Green et al. 2007; Nielson et al. 2009; Mandalari et al. 2013; Sanz & Luyten 2006). Of many potential interactions occurring in foods, the ability of polyphenols to bind to protein and facilitate their denaturation and precipitation has been subject of intensive investigation (Batista et al. 2009; Bohin et al. 2012; De Freitas & Mateus 2001; Pascal et al. 2007; Poncet-Legrand, 2007; Sharma et al. 2008; Soares et al. 2007; Staszewski et al. 2011; Wang et al. 2010; Yuksel et al. 2010). While well known to contribute to the general astringent response characteristic of

polyphenol rich foods (Siebert et al. 2011; Kennedy et al. 2006; Vidal et al. 2004; Misnawi et al. 2004; Troszyńska et al. 2006), these interactions are believed to alter both polyphenol and protein stability in foods and beverages, thereby affecting their availability for chemical reactivity, subsequent digestive release/stability in the gut (bioaccessibility) and potential for intestinal absorption and metabolism by the body (bioavailability) (Arts et al. 2002; López de Lacey et al. 2012; Naz et al. 2011; Niseteo et al. 2012; Serafini et al. 2003; Sharma et al. 2008).

When considering prevalence for protein-polyphenol interactions, several common foods/beverage systems have been studied including beer and wine hazing (Gramshaw et al. 1970; Goertges 1982; Kallithraka et al. 2011; Rinaldi 2012), grain tannins (Emmambux & Taylor 2003; Amarowicz et al. 2005; Mullins & Lee 1991) and milk co-formulated with cocoa and tea (Xie et al. 2012; Green et al. 2007; van der Burg-Koorevaar et al. 2011; Reddy et al. 2005; van het Hof et al. 1998; Hollman et al. 2001; Kyle et al. 2007; Keogh et al. 2007; Roura et al. 2007; Schramm et al. 2003; Serafini et al. 2003; Neilson et al. 2009). Of these foods, tea remains one of the most significant dietary sources of polyphenols in the US diet (Song et al. 2008; Wang et al. 2011; Chun et al. 2007) and its consumption has been associated with prevention and/or amelioration of several chronic and degenerative diseases including cancer (Yuan et al. 2007; Hung et al. 2004; Bonner et al. 2005), cardiovascular disease (Hooper et al. 2008; Zuchi et al. 2010; Steptoe et al. 2007), diabetes (van Dieren et al. 2009; Song et al. 2005) and neurodegenerative processes (Kuriyama et al. 2006; Ng et al. 2008; Hu et al. 2007). Tea is a rich source of flavan-3-ols or catechins, specifically epicatechin (EC), epigallocatechin (EGC), epigallocatechin-gallate (EGCG), and epicatechin-gallate (ECG).

Interestingly, tea is also commonly consumed with addition of protein rich milk in Ireland, Canada, and the UK (Devine et al. 2007; Yang et al. 2000; Weisburger et al. 1997; Hertog et al.1997) and increasingly in other countries including the US. Several studies have demonstrated the ability of flavan-3-ols to interact with individual milk proteins, with these interactions having potential to alter their biological activities (Arts et al. 2002; Lorenz et al. 2007) and bioavailability (Shpigelman et al. 2012). Hydrophobic interactions between bovine milk proteins and polyphenols occur (Yuksel et al. 2010) that are structurally specific and increase with galloylation (i.e. stronger for EGCG and ECG than for EC and EGC) (Xiao et al. 2011).

Effect of milk addition to tea flavan-3-ol bioaccessibility and bioavailability has been previously explored both *in vivo* and *in vitro*, however results to date have been inconsistent and remain controversial. For example, addition of milk at 5.6-40% had no affect on black tea flavan-3-ol bioaccessibility (van der Burg-Koorevaar et al. 2011), however others have demonstrated that addition of milk between 10 and 50% impact flavan-3-ol bioaccessibility from black and green tea *in vitro* (Xie et al. 2012; Green et al. 2007). However, addition of milk to black tea had no apparent affect on levels of blood flavan-3-ol *in vivo* (Van het Hof et al.1998; Kyle et al. 2007). While interactions between flavan-3-ols and protein are well documented, it is unclear the extent to which these polyphenol-protein interactions may impact availability of flavan-3-ols for reaction and absorption in the gut. Furthermore, information on the effect of specific milk proteins on digestive release and stability in the gastrointestinal tract is lacking. Additional insight is needed in order to better understand the potential physiological consequences of

associations between individual milk proteins with flavan-3-ols in order to leverage potential effects for optimal delivery of dietary polyphenols.

The overall objective of this thesis is to develop a more detailed understanding of how interaction between select green tea flavan-3-ols and milk proteins can alter their bioaccessibility from model beverage systems. The purpose of this literature review is to characterize predominant flavan-3-ol components present in green tea, review their associated health benefits and summarize the current state of understanding relative to their bioavailability, metabolism and biological activity as influenced by protein.

1.2 Classification and Nomenclature of Polyphenol Compounds

Polyphenols are plant metabolites synthesized through the shikimate and acetate pathways (Strack D. 1997). Characterized as secondary metabolites, polyphenols aid in plant growth and reproduction as well as serve to protect the plant from pathogens and predators. In addition, many classes of polyphenols also serve as substrates for enzymatically driven browning reactions and can function as antioxidants in plants (Robards et al. 1999).

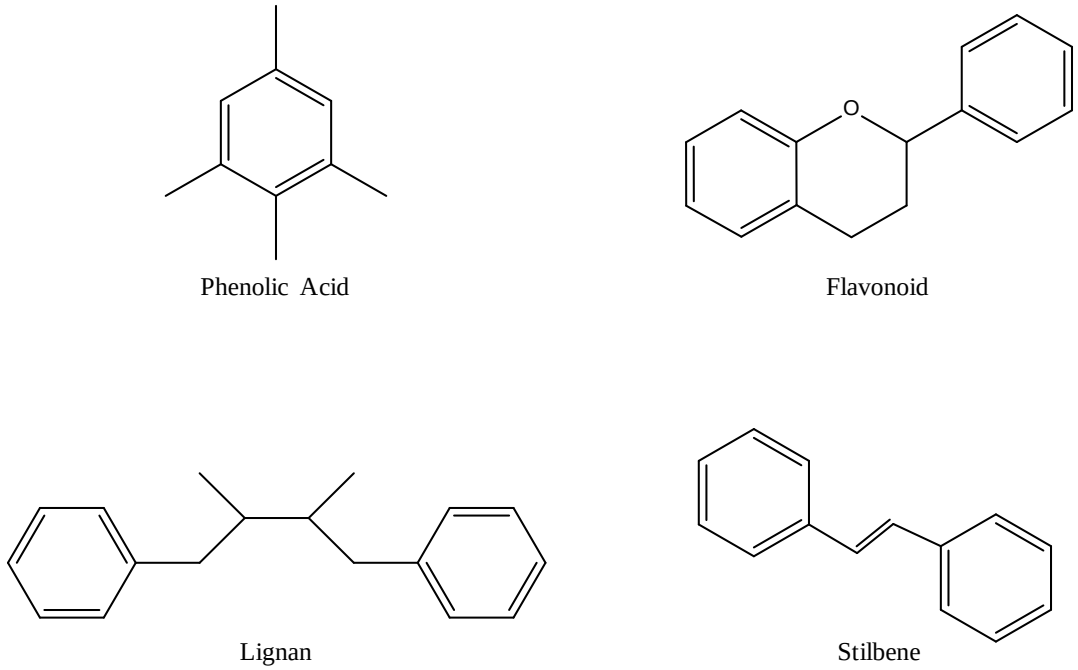
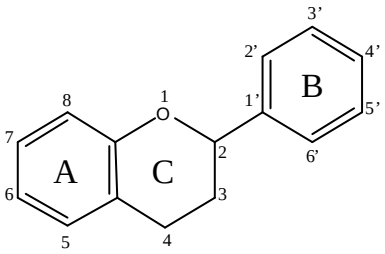
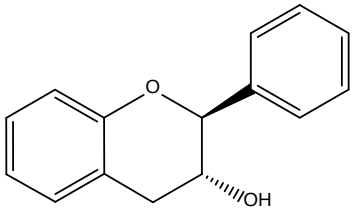


Figure 1: Major phenolic classes of plant secondary metabolites.

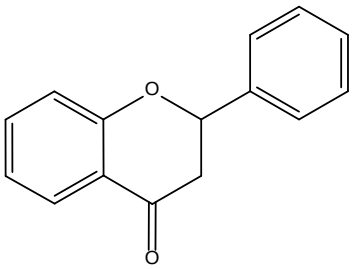
Over 8,000 phenolic compounds have been documented in nature (Tsao R. 2010). The four main classes of polyphenols include phenolic acids, flavonoids, stilbenes, and lignans (Scalbert et al. 2000) (Figure 1). Phenolic rings containing one or more hydroxyl substituents are characteristic of most phenolic structures. Simple phenolic acids can be divided into two main types, benzoic acid and cinnamic acid derivatives, based on C1–C6 and C3–C6 backbones. Two major phenolic acids are caffeic acid, found in fruits, vegetables and coffee, and ferulic acid, typically found in the bran fraction of cereal grain (Scalbert et al. 2000). Flavonoids are more complex polyphenols having a C6–C3–C6 backbone in which the two C6 units are of phenolic nature (Tsao R. 2010). Flavonoids can be further divided into different sub-groups according to the oxidation state of the heterocyclan pyran ring (Cheynier 2005). Major flavonoid classes include anthocyanins, flavan-3-ols, flavones, flavanones and flavonols (Figure 2).



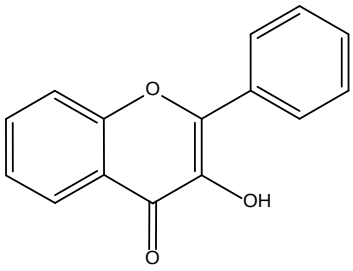
Basic flavonoid structure



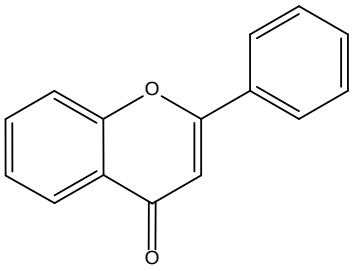
Flavan-3-ol



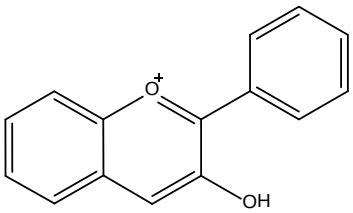
Flavanone



Flavonol



Flavone



Anthocyanidin

Figure 2: Major flavonoid sub-classes

Flavan-3-ols are believed to be the most abundant flavonoid found in the western diet (Chun et al. 2007). They are present in numerous common foods and beverages including tea, apple, cocoa and grape derived products (Hara et al. 1995; Gu et al. 2004) (Table 1). Flavan-3-ols are structurally characterized by a lack in each a double bond between C2 and C3, a C4 carbonyl, and of conjugation to glycosidic moiety at C3 (as most other classes of flavonoids naturally are). These unique structural characteristics provide flavan-3-ols the ability to have two chiral centers and four diasteroisomers (Tsao 2010). Furthermore, monomeric catechin and epi-catechin are known to be precursours for more complicated polymers referred to as proanthocyanidins (PACs) ranging in degree of polymerization from DP2 to about DP30 (Takahata et al. 2001; Hayasaka et al. 2003; Hanlin et al. 2011). The eight major flavan-3-ol monomers that occur in nature and predominate in green tea include (-)-gallocatechin-3-gallate (GCG), (-)-epigallocatechin-3-gallate (EGCG), (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (+/-)-catechin (C), (-)-epicatechin (EC), (-)-catechin-3-gallate (CG) and (-)-epicatechin-3-gallate (ECG). The 5’ position on the B ring of EGC is a pyrogallol group, compared to the pyrocatechol group on the same position of EC. ECG and EGCG are gallic esters of EC and EGC, respectively, containing a gallic acid moiety at C3 (Henning et al. 2008).

Table 1: Flavonoid content of selected foods.

Food	Number of data points	Mean concentration (mg/100g, edible portion) +/- SEM						
		(-) –EC	(-) -ECG	(-) -EGC	(-) -EGCG	(+) -C	(+) -GC	Total
Apple, raw, with skin	59 – 150	7.53± 0.44	0.01± 0.00	0.26± 0.07	0.19± 0.11	1.30± 0.07	-	9.29± 0.46
Apple juice	13	4.71± 2.25	-	-	-	1.25± 0.69	-	5.96± 2.35
Applesauce	1	5.41± 5.41	-	-	-	0.69± 0.69	-	6.10± 5.45
Cocoa, dry powder	13	196.43± 45.38	-	-	-	64.82± 14.53	-	261.25± 47.65
Candies, chocolate, dark	5	84.40± 13.54	-	-	-	24.20± 5.74	-	108.60± 14.71
Candies, chocolate, milk	6 or 9	10.88± 2.68	-	-	-	4.16± 1.21	-	15.04± 2.94
Grapes, black	4 or 11	8.68± 2.48	2.81± -	-	-	10.14± 2.91	-	21.63± 3.82
Grape juice	25 – 28	0.54± 0.07			-	0.79± 0.09	-	1.33± 0.11
Grapes, white or green	7 or 14	1.70± 0.42	0.25± 0.08	0.02± 0.00	-	3.73± 0.92	0.01± 0.00	5.71± 1.01
Tea, black, brewed	55 – 94	2.13± 0.10	5.86± 0.17	8.07± 0.45	9.36± 0.46	1.51± 0.07	1.25± 0.22	28.18± 0.71
Tea, green, brewed	66 - 97	7.36± 0.31	16.39± 1.93	22.27± 0.89	64.15± 5.02	3.28± 0.88	1.54± -	114.99± 5.53
Tea, green, ready-to-drink	2	1.98± 0.11	0.93± 0.06	4.99± 0.53	3.96± 0.40	0.02		11.88± 0.68
Tea, white, brewed	3	-	9.20± -	19.40± -	46.00± -	-	-	74.60± 0.00

Adapted from: U.S. Department of Agriculture, Agricultural Research Service. 2011. USDA. Database for the Flavonoid Content of Selected Food.

1.3 Polyphenol Biosynthesis

Phenylalanine, a product of the shikimate pathway is a precursor for the majority of phenolic compounds found in plants. The biosynthesis of polyphenolic compounds is outlined in Figure 3. General polyphenol metabolism involves conversion of phenylalanine to *p*-coumaric acid (hydroxycinnamic acid). Key enzymes catalyzing this transformation include phenylalanine ammonialyase (PAL), cinnamate 4-hydroxylase (C4H), and hydroxycinnamate CoA ligase (CoAL). PAL catalyzes deamination of phenylalanine to *trans*-cinnamic acid followed by hydroxylation and methylation reactions catalyzed by monooxygenases to form hydroxycinnamates. Hydroxycinnamate:CoA ligase or O-glucosyltransferase then activate hydroxycinnamates. Resulting hydroxycinnamate CoAs enter phenylpropanoid reactions including: condensation with malonyl-CoA leading to flavonoids; degradation by removal of an acetate unit resulting in hydroxybenzoates; NADPH-dependent reduction leading to lignin precursors and hydroxycinnamyl alchohols; and conjugation through attachment to a hydroxyl or amino-group-bearing molecule with loss of water leading to esters, amides, or in rare cases attachment to phenolic hydroxyl groups leading to glycosides (Strack D. 1997).

Flavonoid biosynthesis is initiated by chalcone synthase, which synthesizes the basic C_{15} skeleton by catalyzing condensation of 4-coumaroyl-CoA to form 2',4,4',6'-tetrahydroxychalcone. Naringenin chalcone, possessing the characteristic B-ring of flavonoids, is the first intermediate product. Acetyl-CoA carboxylase supplies Malonyl-CoA providing acetate units that randomly orient to form the flavonoid A-ring, leading the 5-hydroxyflavonoid pathway. Flavonoids usually occur as water-soluble glycosides,

commonly 3-O-(di)glycosides (Strack D. 1997). Exceptions include some polymethoxylated derivatives and flavan-3-ols.

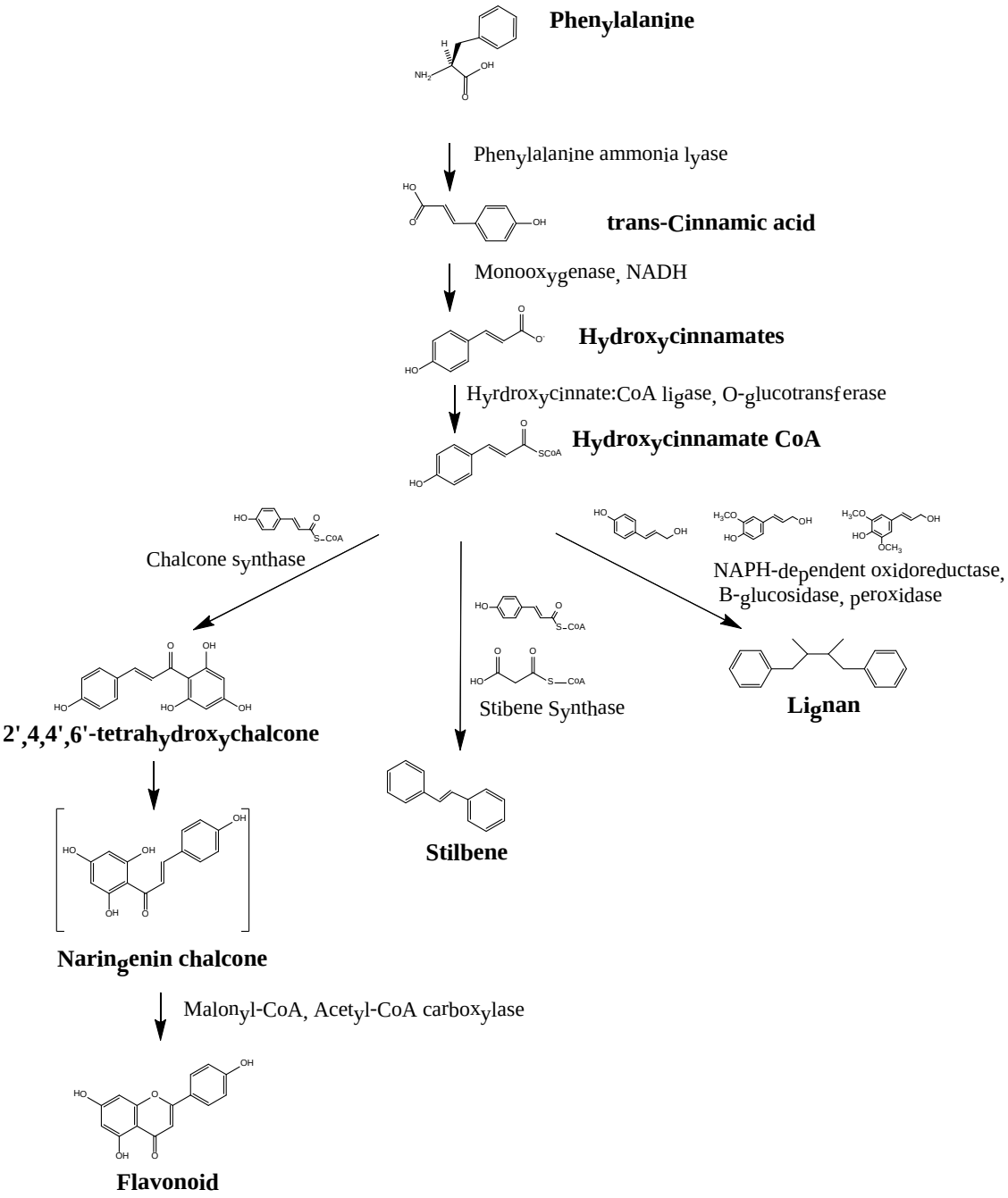


Figure 3: Biosynthesis of major polyphenolic compounds

Ashihara et al. 2010 suggested possible biosynthetic pathways of specific flavan-3-ols. Chalcone isomerase transforms naringenin-chalcone into naringenin, which is then converted to dihydrokaempferol (DHK) by flavanone 3-hydroxylase. From here there are two pathways that may occur: one resulting in EGC and EGCG, and the other in EC and ECG. DHK is converted to either dihydromyricetin or dihydroquercetin by flavonoid 3',5'-hydroxylase or flavanone 3-hydroxylase, respectively, followed by sequential enzymatic transformations by dihydroflavanol 4-reductase (DFR), anthocyaninin synthase (ANS), and anthocyanidin reductase (ANR). EGC results from enzymatic transformation of dihydromyricetin, and EC from dihydroquercetin. EGC and EC may then become esterified to gallic acid at the C3 position to form EGCG and ECG, respectively.

1.4 Flavan-3-ols and Health

1.4.1 Cardiovascular Disease

Cardiovascular diseases (CVDs) are major contributors to fatality worldwide. Major causes of CVDs are believed to include oxidized low density lipoprotein (LDL) and free radical damage to the endothelium which ultimately results in inflammatory stress and subsequent damage to endothelial structure. Stiffness and blockages may then develop in vessels leading to increased risk for heart attacks, stroke or other forms of coronary artery disease (Mendis et al. 2011).

Many studies have suggested a protective association between dietary polyphenol consumption and CVD risk and outcomes. Intermediate intake (mg/day) of flavonoid classes including anthocyanidins, flavan-3-ols, flavones, flavonols, and

proanthocyanidins were associated with a lower risk of death from CVD (McCullough et al. 2012). Furthermore, meta-analysis of randomized controlled trials indicated isoflavone and flavan-3-ol rich foods specifically including chocolate, soy protein isolate, and green tea significantly reduce cardiovascular risk factors (Hooper et al. 2008) and supplementation of 200 mg/day of grape seed flavan-3-ols resulted in a significant improvement in overall vascular health (Weseler et al. 2011). In addition, lag time in LDL oxidation *ex vivo* significantly increased in healthy men consuming an equivalent of seven to eight cups a day of green tea for two weeks (Miura et al. 2000), however consumption of six cups of green or black tea per day for four weeks showed no effect on LDL oxidation *in vivo* or *ex vivo* (van het Hof et al. 1997), indicating that tea flavonoids may have more of an effect on onset than extent of LDL oxidation.

Risk of heart attack is increased by adherence and aggregation of blood platelets to the vascular wall (Gregg & Goldschmidt-Clermont 2003). Studies suggest that flavan-3-ol-rich supplements and select flavonoid rich foods may decrease platelet aggregation. Consumption of flavan-3-ol rich dark chocolate (40 g) by heart transplant recipients decreased platelet adhesion in correlation to increased serum epicatechin, and improved coronary vasomotion (Flammer et al. 2007). In addition, ADP- and epinephrine-stimulated platelet activity of plasma from male smokers was decreased *ex vivo* following consumption of a flavan-3-ol-rich supplement (Polagruto et al. 2007).

1.4.2 Cancer

Cancer is caused by uncontrolled growth and spread of abnormal cells that often leads to death. In 2007, the burden of direct medical costs and indirect mortality was

about $226.8 billion and in 2008, about 12 million Americans had a history of cancer. (American Cancer Society 2012).

Results from epidemiological studies concerning effect of flavan-3-ols on cancer have been inconclusive for the most part. Flavan-3-ol rich green tea has been both negatively associated (Wang et al. 2007; Hung et al. 2004) and unassociated (Wang et al. 2002) with esophageal cancer and associated both negatively (Zhong et al. 2001; Bonner et al. 2005) and positively (Tewes et al. 1990) with lung cancer risk. Furthermore, in the Shanghai Cohort Study EGC and its methylated metabolite, but not EC nor its metabolites, were significantly associated with colon cancer protection in men (Yuan et al. 2007). Confounding variables such as temperature of the tea and smoking status of participants may complicate analysis of data in esophageal and lung cancer studies, respectively (Gao et al. 1994; Yuan et al. 2011).

1.4.3 Diabetes

Diabetes is the 7th leading cause of death in the United States, affecting about 8.3% of the population (Centers for Disease Control and Prevention 2011). The metabolic disorder is characterized by hyperglycemia due to defects in insulin secretion and/or action (American Diabetes Association2008).

Similar to studies on association of flavan-3-ol effects with cancer, studies exploring effect of flavan-3-ol consumption on diabetes have been inconclusive. Coffee and tea consumption (3 cups/day) reduced risk of type 2 diabetes by about 42% in one study (van Dieren et al. 2009), however in another there was no significant association

between diabetes risk and consumption of coffee or tea, except in subjects over 60 years old who had lost weight (Greenberg et al. 2005).

1.4.4 Neuroprotection

Cognitive deterioration during the ageing process is a major concern. Two major diseases characterized by cognitive decline are Alzheimer's disease (AD) and Parkinson's disease (PD). One in eight Americans suffer from AD, an ailment caused by accumulation of amyloid peptides and tau protein in brain neurons (Alzheimer's Association 2012). PD is a neurodegenerative disease caused by dysfunction of dopaminergic neurons (Schapira et al. 1990; Parker et al. 2008; Keeney et al. 2006) and affects about 1 in 100 people (Shapira 2004). Longitudinal and cross-sectional studies found that total tea intake by Chinese, Japanese, and Finnish adults was significantly associated with lower prevalence of cognitive decline (Ng et al. 2008; Kuriyama et al. 2006; Hu et al. 2007).

Evidence from numerous animal studies further supports that flavan-3-ols may have neuroprotective benefits. EGCG (50 mg/kg) administered orally through drinking water for 6 months reduced amyloid β peptides and modulated tau profiles in Alzheimer transgenic mice (Rezai-Zadeh et al. 2008). The ability of green tea polyphenols (GTPs) to improve stress induced cognitive impairment was studied. Male Sprague–Dawley rats consuming ≥0.5% GTPs in their diet showed improved psychological stress-induced cognitive function compared to controls (Chen et al. 2009). Furthermore, GTPs (150 and 450 g/day-fed, 7 days) protected dopaminergic neurons, decreased generation of reactive

oxygen species and nitric oxide, and inhibited lipid peroxidation in Male Sprague–Dawley rat models of PD (Guo et al. 2007).

It is important to note that results presented above have been inconsistent. Variability in food, food component and polyphenol doses (low to high supplemental doses) between studies may contribute to these observations. Each type of study has potential drawbacks. In epidemiological studies, correlations, but not causation, can be made between intake and disease. Furthermore, the complexity of the human diet makes it difficult to isolate the health effect of individual dietary phenols (Hollman et al. 2001) or their biologicaly relevant metabolites. Differences in agricultural practices, season, processing, portion of food used, or amount of food or food component used all serve to alter flavan-3-ol exposure and perhaps outcomes observed in epidemiological and experimental settings (Clifford et al. 2004).

1.4.5 Protective Mechanisms Associated with Flavan-3-ols

Reactive oxygen, nitrogen, and halide species in amounts that overwhelm antioxidant defense systems result in oxidative stress that lead to damage to cells and change cell signaling pathways, initializing ageing and disease processes (Kontic-Vucinic et al. 2008; Wang et al. 2006a; Pong 2003). The health-promoting activity most commonly associated with flavan-3-ols has been their antioxidant activity (Halliwell et al. 2005) derived from the multi-phenolic structure that allows polyphenols to reduce reactive oxygen species by donating an electron transforming into an aryloxyl radical with an unpaired electron stabilized over its aromatic rings (Dangles et al 2012). These radicals can prevent initiation of new chain reactions and can act as terminators of the

propagation route by reacting with other free radicals (Shahidi et al 1992). Polyphenols can also indirectly act as antioxidants by chelation of divalent metal ions involved in pro-oxidative reactions including Fenton and Haber-Weiss reactions (Duthie et al 2000) or by stimulating the body's endogenous antioxidant systems (Li et al. 2006; Chandronitha et al. 2010; Yuan et al. 2007).

Flavan-3-ols have demonstrated the ability to regenerate endogenous antioxidants and stimulate defense mechanisms of cells by up-regulating expression of antioxidant enzymes (Dangles et al. 2012). As little as 0.05 μM of procyanidins from grape seed extract enhanced endothelial NO-synthase-mediated relaxation in internal mammary aortic rings, indicative of cardioprotective effects (Aldiniet al. 2003). In addition, a cocao extract inhibited CYP1A activity reducing the mutagenicity of benzo[a]pyrene by limiting its biotransformation to the ultimate carcinogenic form (Ohno et al. 2009). Apple polyphenols including C and EC induced glutathione S-transferase, a phase II gene involved in xenobiotic detoxification, in colon epithelial cells (Petermann et al. 2009). In a study by Manna et al. 2009, tea polyphenols restricted benzo[a]pyrene-induced lung carcinogenesis in mice indicated by altered expression of p53-associated genes. Furthermore, EGCG increased protein levels of Nrf2 and mRNA levels of UGTs in nude mice, suggesting EGCG activates the Nrf2-UGT1A pathway (Yuan et al. 2007).

Flavan-3-ols have also demonstrated the ability to modulate cell-to-cell communication by upregulating intracellular gap junction communication. For example, cocoa flavan-3-ols including EC and C have been shown to attenuate hydrogen peroxide-induced inhibition of gap-junctional intercellular communication (GJIC) in rat liver

epithelial cells through antioxidant activity and direct inhibition of mitogen-activated protein kinase (MEK) 1 activity (Lee et al. 2008).

1.5 Overview of Bioaccessibility and Bioavailability of Flavan-3-ols

While many biological activities have been proposed and explored for flavan-3-ols, it is important to consider the ability of these individual species to cross the intestinal barrier and be made availabile for tissue uptake and utilization. Therefore bioavailability of flavan-3-ols has been increasingly a topic of focused research and subject of many comprehensive reviews. The term *bioaccessibility* is used to refer to the pre-absorptive/digestive release and solubilization of compounds from food matrices by digestive enzymes or bacterial microflora present in the gut. Compounds that are stable to gut luminal condition (e.g. enzymes, pH and oxidative environment) during digestion (*digestive stability*) are described as available for subsequent absorption in the intestine (bioaccessible) (Saura-Calixto et al. 2007) (Figure 4). The stability of flavan-3-ols during digestion as well as their metabolism by mammalian phase I and phase II systems and transformation by microbiota are subject of extensive reviews (Spencer et al. 2003; Ferruzzi et al. 2012; Monagas et al. 2010). This review will primarily highlight key aspects of oral, gastric and intestinal processing as it related to flavan-3-ol bioaccessibility and is the focus of the research described in subsequent chapters.

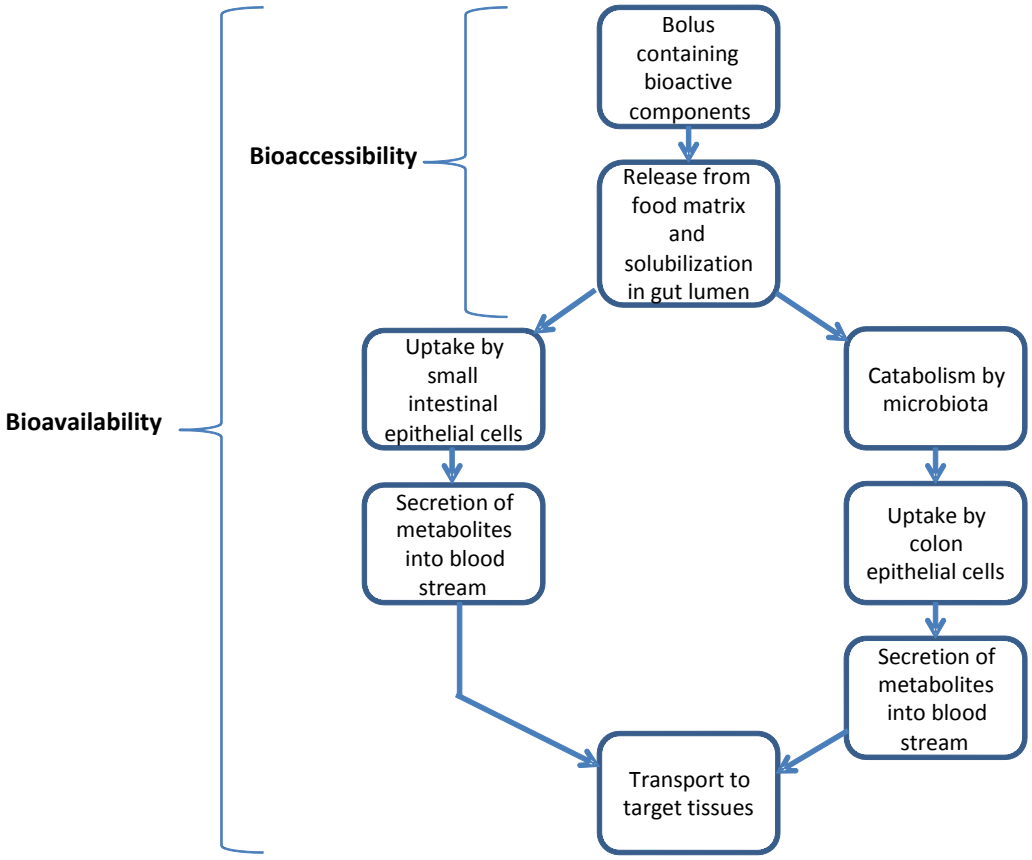


Figure 4: A schematic outlining the concept of bioaccessibility and bioavailability of bioactive components after consumption.

1.5.1 Oral Phase

Mechanical break-down of the food matrix and enzymatic degradation of carbohydrates by salivary α-amylase at near neutral pH occurs during the oral phase of digestion. Flavan-3-ol-protein interactions have been well described in this phase of digestion. Proline-rich salivary proteins have demonstrated the ability to interact with flavan-3-ol monomers and polymers resulting in an “astringent” response (Bajec et al. 2008; Bate-Smith 1954; Lu et al. 1998). Interestingly, studies have demonstrated the

ability of these interactions to stabilize flavan-3-ols (Tsuchiya 1997) and prevent association of flavan-3-ols with digestive enzymes, mitigating the potential antinutritive effect associated with complex polyphenol polymers (tannins) (Lu et al. 1998; Prinz et al. 2000) further down the GI tract. Additional modifications to flavan-3-ols during the oral phase may include potential for degalloylation of EGCG to EGC by esterase activity in human saliva (Yang et al. 1999). However, this notion remains controversial as the presence of esterases in saliva remains a topic of intense debate. In general, oral processing is not believed to induce significant changes to flavan-3-ol structure or availability other than through interaction of free flavan-3-ols with salivary proteins.

1.5.2 Gastric Phase

Orally processed food or beverages containing flavan-3-ols is passed from the oral cavity into the stomach where digestion of the food matrix continues in the gastric environment. Mechanical break-down by mixing as well as initial digestion of the food matrix by pepsin occurs at low pH during this phase. Monomeric flavan-3-ols are generally water soluble and stable to low pH (2-4) (Spencer et al. 2000; Uzunović & Vranić 2008), and thus begin to be extracted from the partially digested food matrix and solubilized in the stomach during chyme formation. *In vitro* studies suggest that after solubilization, flavan-3-ol polymer proanthocyanidins (PACs) may be hydrolyzed to smaller units or monomers (Spencer et al; 2000, Kahle et al. 2011; Rios et al. 2002). However, as with esterase activity, evidence of depolymerization of PACs in the stomach remains limited and it is generally believed that flavan-3-ols in all forms remain generally stable to gastric conditions (Fernández et al. 2013; Bouayed et al. 2012; Laurent et al.

2007). While certain phenolics/polyphenols, such as anthocyanin, quercetin, chlorogenic acid and phenolic acids, may be absorbed in the gastric environment (Passamonti et al. 2005; Konishi et al. 2006; Lafay et al. 2006; Crespy et al. 2002; Vanzo et al. 2007), most flavonoids, including flavan-3-ols, are not absorbed in this portion of the GI tract to any significant extent. However, proposed mechanisms for absorption of polyphenols in the stomach include passive trans-epithelial diffusion (Tsuji et al. 1996) and carrier-mediated trans-epithelial diffusion (Vanzo et al. 2007) as well active transport by billitranslocase (Passamonti et al. 2002)

1.5.3 Small Intestine

Gastric chyme containing partially digested food and partially bioaccessible flavan-3-ols is released from the stomach into the upper small intestine (duodenum) for further digestion. Bile salts, pancreatic enzymes including lipase, trypsin, α-amylase, and elevated pH (pH 6-7.5) (Fallingborg 1999) are characteristic of small intestinal conditions. Flavan-3-ols appear to be susceptible to oxidative degradation at this stage of digestion, possibly due to oxidative processes occurring in the presence of both reactive oxygen species and elevated pH conditions (>5.5) (Yoshino et al. 1999; Zhu et al. 2002a; Green et al. 2007; Neilson et al. 2007; Ferruzzi et al. 2010). Additionally, flavan-3-ols may epimerize during the duodenal phase (Kahle et al. 2011) generating higher proportions of C, GC and GCG forms from predominant epi forms in food and beverages.

Intestinal absorption of polyphenols and flavan-3-ols has been subject of extensive reviews (Scalbert et al. 2000; Manach et al. 2004; Monagas et al. 2010). Extent of flavan-3-ol absorption and bioavailability may differ according to stereochemistry

(Baba et al. 2000) and galloylation (Auger et al. 2008; Stalmach et al. 2009; Stalmach et al. 2010; Lee et al. 2002). Absorption from the small intestine is limited to flavonoid aglycones such as flavan-3-ols and select O-β-D-glucosides (Dangles et al. 2012). O-β-D-glucosides can be hydrolyzed by lactase phlorizin hydrolase to its aglycone form and absorbed via passive diffusion as a result of its increased lipophilicity and its proximity to the cellular membrane (Day et al. 2000). Absorption directly through a sodium-dependent glucose transporter (SGLT1) intact (Walgren et al. 2000; Wolffram et al. 2002) as well as intracellular hydrolysis by cytosolic β-glucosidase have been demonstrated (Day et al. 2003). Flavan-3-ols are the flavonoids that are most commonly present as aglycosides in the diet, therefore their monomers may be absorbed directly in the small intestine for further metabolism by passive or carrier-mediated diffusion (Henning et al. 2008). Enzymes active in transport of flavan-3-ols include monocarboxylate transporter (MCT) and organic anion transport protein B (OATP-B). OATP-B is a pH dependent transporter that aided in passive absorption of quercetin into small intestine epithelial cells *in vitro* (Chabane et al. 2009). MCT1 catalyzes proton-coupled transport of flavonoids. ECG was transported *in vitro*, and multiple flavonoid agylcones were transported both *in vitro* and *in vivo* by MCT1 (Vaidyanathan & Walle 2003; Wang 2007). Generally, monomeric flavan-3-ols reach T_{max} in plasma at 1.5 hours after ingestion (Manach et al. 2005). However flavan-3-ol oligomers and polymers with degree of polymerization over three are not believed to be absorbed effectively and are passed to the colon where further metabolism by colonic microbiota is possible (Manach et al. 2004; Donovan et al. 2006).

Once present in the enterocyte, various xenobiotic “Phase II” metabolizing enzymes including uridine-5’-diphosphate glucotransferases (UGTs) present in the small

intestine, and sulfotransferases (SULT) and catechol-O-methyltransferases (COMT), also present in the liver, begin to biotransform flavan-3-ol aglycones to sulfate, glucuronide, and/or methylated metabolites. These conjugated metabolites are further distributed in circulation and can also be effluxed back into the lumen of the small intestine. UGT1 is the enzyme considered responsible for flavonoid glucuronidation (Cheng et al. 1999). Conjugation of EC occurs at C-3' and C-4' (B ring) and C-5 and C-7 (A ring) hydroxyl groups, resulting in monoglucuronide, sulfate, methyl ester, or combined derivative forms (Baba et al. 2000; Natsume et al. 2003; Schroeter et al. 2006). The main glucuronide derivative of both EC and EGC is 3'-O glucuronide (Natsume et al. 2003; Li et al. 2001; Lu et al. 2003; Urpi-Sarda et al. 2009). However, after consumption of cocoa, O-sulfated metabolites of EC have been reported as predominant urinary metabolites (Roura et al. 2008) and following consumption of tea, EGC metabolites identified include 3'- and 7-O glucuronides and 4'-O methyl and its derivatives (Sang et al. 2008a; Sang et al. 2008b; Li et al 2001; Meng et al. 2002; Lu et al. 2003). EGCG metabolites were primarily methylated on B and D rings in cytosol samples from CF-1 mice, Sprague-Dawley rat, and human liver and in biliary fluids of Wistar rats (Lu et al. 2003; Kida et al. 2000). Prominent EGCG metabolites found in human and rat sera include 4',4"-di-O-methylEGCG, and 4"-O-methylEGCG (Meng et al. 2002). Interestingly, EGCG may also reach plasma unmetabolized: it was reported as the only unmetabolized flavan-3-ol from ready-to-drink green tea recovered in human plasma, and was present at higher concentrations than EGC and EC metabolites (Del Rio et al. 2010).

With regards to procyanidins, dimeric procyanidins have been detected at very low concentrations (nM range) in human plasma after consumption of cocoa and grape

seeds (Holt et al. 2002; Sano et al. 2003). Both procyanidin dimers and trimers have also been detected in Sprague-Dawley and Wistar rat plasma after ingestion of cocoa extracts (Baba et al. 2002; Zhu et al. 2002b) and cocoa cream and hazelnut skin extracts (Serra et al. 2013). Also, only native and methylated procyanidin dimer metabolites were found in Wistar rat plasma (Shoji et al. 2006). However, in general, bioavailability of dimeric forms was reported to be significantly lower than monomeric flavan-3-ol forms.

The adenosine triphosphate (ATP)-binding cassette (ABC) family of transporters including multidrug resistance protein (MRP) and P-glycoprotein (P-gp) are responsible for efflux of polyphenolic metabolites from intestinal enterocytes back to the gut lumen thereby limiting overall trans-cellular transport and oral bioavailability of flavan-3-ols. Metabolites that are passed to the bloodstream may undergo further metabolism in the liver and kidney. Additionally, absorbed or hepatic metabolites may be subsequently recycled to the small intestine via enterohepatic transport in bile (Crozier et al. 2009; Prasain et al. 2007; Clifford et al. 2004). Overall, flavan-3-ols and their conjugated metabolites are generally poorly absorbed in the small intestine, and as a consequence, their concentrations in plasma rarely exceed 1 μM (Manach et al. 2005). However, intestinal tissues do appear to be a main site for their accumulation as indicated by animal studies (Suganuma et al. 1998; Janle et al. 2010; Swezey et al. 2003).

1.5.4 Colon

About 90-95% of ingested polyphenols reach the colon after consumption (Clifford et al. 2004) where they are extensively metabolized by colonic microbiota (Dangles et al. 2012). The microbiota contain glycosidases that are able to

cleaveconjugated moieties to aglycones, which are then subjected to subsequent ring fission and other metabolic conversion. In general these processes lead to production of smaller molecular weight benzoic acid, hydroxycinnamate and other phenolic acid derivatives (Crozier et al. 2009). Millimolar concentrations of benzoic acid, phenylacetic acid, and phenylpropionic acid derivatives can result from this catabolism (Williamson & Clifford, 2010) and have been subsequently detected in urine of animals treated with polyphenols (Sang et al. 2008; Crozier et al. 2009).

Galloylated flavan-3-ols are catabolized first via cleavage of gallic aid, followed by decarboxylation of gallic acid to pyrogallol (Meselhy et al. 1997; Kohri et al. 2003; Roowi et al. 2010) and opening of the C-ring. Subsequently, conversions to γ-valerolactone (Meselhy et al. 1997; Roowi et al. 2010, Groenewoud & Hundt 1984) and valeric acid (Kohri et al. 2003) followed by transformation of valeric acid to hydroxyphenylpropionic and hydroxybenzoic acid via β-oxidation may occur. Catabolized flavan-3-ols are then metabolized in the liver by phase II enzymes and subsequently eliminated in urine or feces. Interestingly, a recent study showed that after green tea consumption, polyhydroxyphenyl-γ-valerolactones were present at 10x the concentration in urine than any other metabolites (Sang et al. 2008).

These microbial metabolites may also be absorbed, distributed through the body and ultimately excreted in urine at much higher quantities than the parent flavan-3-ol and primary phase II metabolites absorbed in the small intestine (Crozier et al. 2009). Evidence shows benzoic, salicylic, *p*-coumaric, and ferulic acids may be transported actively by monocarboxylate transporter MCT1 (Tsuji et al. 1994; Takanaga et al 1994; Konishi et al. 2003), the majority of these being subjected to mammalian conjugation by

Phase II enzymes in the gut and liver (Clifford et al. 2004). In addition, carrier-mediated transport was involved in absorption of ferulic acid in an *in vitro* model for colonic epithelium (Poquet et al. 2008).

1.6 Food Matrix Interactions

While bioavailability of polyphenols is reported to be poor, the notion that this limited absorption can be modulated by specific food matrix interactions has been subject of several lines of investigation. Specifically, factors that alter stability in foods and beverages, their digestive release and stability in the gut (bioaccessibility) or their transport/metabolism by the gastrointestinal epithelium may all provide avenues to positively affect flavan-3-ol bioavailability (Parada & Aguilera 2007; Neilson & Ferruzzi, 2011). While polyphenol-micronutrient and polyphenol-polyphenol interactions have been subject of several investigations, polyphenol-polysaccharide and polyphenol-protein interactions have also demonstrated several specific effects on polyphenol bioavailability. Considering the focus of this thesis on flavan-3-ol-protein interactions, the influence of these macronutrients is therefore the focus of this section.

1.6.1 Polysaccharide-Polyphenol Interactions

Phenolic and polyphenolic compounds in plants can be defined as extractable (EPP), or non-extractable (NEPP) (Chandrasekara & Shahidi, 2011). NEPP's mainly form complexes with polysaccharides associated with the cell wall (Ferguson et al. 2001) and are not directly bioavailable in the small intestine (Manach et al. 2005), but can be released in the colon, through fermentation by microbiota and transformed into

absorbable metabolites. These metabolites are typically phenolic acids, such as hydroxyphenylacetic, phenylpropionic, and phenylbutryric acids (Saura-Calixto et al. 2010a; Saura-Calixto et al. 2010; Selma et al. 2009).

While these direct, covalent interactions are well known to limit availability of phenolics, other associations with plant cell walls and polysaccharides may serve to limit bioaccessibility of polyphenols in the gut lumen. For example, flavan-3-ols with a galloyl group (such as EGC and EGCG and ECG) are more able to interact non-covalently with β-glucan fibers than those without (Gao et al. 2012). Additionally, anthocyanins, which are charged species, have been reported to interact with plant cell wall polysaccharides including pectin and lignins after ingestion, likely facilitating their transport to the colon and limiting their absorption in the stomach and small intestine (Padayachee et al. 2012). As such, the majority of anthocyanins only become bioavailable following colonic digestion which includes microbial fermentation of plant polysaccharides and metabolism of flavonoid backbones (Williamson & Clifford, 2010).

In addition to effects of polyphenols in complexation with cell wall material on polyphenol bioavailability, effects related to polyphenol bioactivity have been shown. In a study by Jacob et al. 2008, twenty-five percent of polyphenols from a grape juice concentrate were nondialyzable. These complexes had different anthocyanin conjugates and exhibited reduced radical scavenging activity compared to dialyzate, possibly due to differences in anthocyanin composition and to complex formation. However, non-extractable procyanidins in fruit showed positive correlations with bile acid binding, which has been implicated in lowering cholesterol (Rideout et al. 2008), and radical scavenging activities (Hamauzu & Mizuno 2011). Polysaccharide-polyphenol

interactions may also compete with and thus inhibit interactions between polyphenols and digestive enzymes in the gut lumen. For example, polysaccharides (arabic gum, β-cyclodextrin, and pectins) reduced polyphenol interactions with α-amylase (Soares et al. 2009), an interaction associated with harmful effects such as decrease in body weight gain (Griffiths et al. 1986).

Finally, presence of digestible carbohydrate (starch or sugars) appears to positively influence intestinal uptake and transport of select polyphenols. Uptake of monomeric procyanidins from grape seed procyanidin extracts (300 mg) was enhanced by co-ingestion of a carbohydrate-rich food (600 mg; containing hydrolyzed wheat, wheat, barley, maize, and oats) (Serra et al. 2010). A similar positive effect of carbohydrate on flavan-3-ol absorption was noted in both animal and human clinical studies on tea (Peters et al. 2010) and cocoa (Schramm et al. 2003; Nielson et al. 2009; 2010). However, Maillard reaction products slightly reduced chlorogenic acid absorption (Dupas et al. 2006a). The ability of non-digestible carbohydrates to both associate and limit bioaccessibility of and digestible carbohydrate to potentiate absorption of flavan-3-ols further complicates the design and interpretation of many bioavailability studies. This may be due in part, to direct effects to GI motility, or by association with carbohydrate-flavan-3-ol transporters including SGLUT1, GLUT2 and MCT. However, the extent to which this may actually be the case remains to be assessed.

1.6.2 Protein-Polyphenol Interactions

1.6.2.1 Mechanisms and Factors Affecting Interactions

Polyphenols have been reported to bind to several biologically relevant proteins including common food proteins (milk, soy and cereal), proline rich salivary proteins, digestive enzymes, and plasma proteins. These interactions are believed to potentially influence biological activities and bioavailability of polyphenols. As such, it is not surprising that the mechanims of these interactions have been subject of several studies. Examples of types and strengths of specific interactions between select proteins and polyphenols are summarized in Table 2. Central to these interactions is hydrophobic stacking of aromatic amino acid side chains within protein to those of dipoles within polyphenols generated by London forces (Yuksel et al. 2010). Combined with hydrogen bonding interaction of hydroxyl groups of polyphenols with protein chains these forces have been reported to be major driver of such interactions. Both of these interactions may induce changes in protein structure, depending on the protein involved (Hagerman et al. 2012).

Table 2: Binding kinetics and mechanisms of protein-(poly)phenol interactions.

Protein	Polyphenol	pH	Binding Affinity (K)	Binding Mechanism	Reference
α-casein	(+)-C	7.4	$1.8x10^3M^{-1}$	Primarily London dispersion forces, some hydrogen-bonding	Hasni et al. 2011
	(-)-EC	7.4	$1.8x10^3M^{-1}$		
	(-)-EGC	7.4	$2.4x10^3M^{-1}$		
	(-)-EGCG	7.4	$7.4x10^3M^{-1}$		
β-casein	(+)-C	7.4	$2.9x10^3M^{-1}$	Primarily London dispersion forces, some hydrogen-bonding	Hasni et al. 2011
	(-)-EC	7.4	$2.5x10^3M^{-1}$		
	(-)-EGC	7.4	$3.5x10^3M^{-1}$		
	(-)-EGCG	7.4	$1.59x10^4M^{-1}$		
β-lactoglobulin	(+)-C	7.4	$2.2x10^3M^{-1}$	London dispersion forces, hydrogen-bonding	Kanakis et al. 2011
	(-)-EC	7.4	$3.2x10^3M^{-1}$		
	(-)-EGC	7.4	$1.1x10^4M^{-1}$		
	(-)-EGCG	7.4	$1.3x10^4M^{-1}$		
α-amylase	(+)-C	5	$1.4x10^4M^{-1}$	London dispersion forces, hydrogen-bonding	Soares et al. 2007
	(-)-ECG	5	$2.0x10^4M^{-1}$		
	procyanidin B2 gallate	5	$2.2x10^4M^{-1}$		
	procyanidin B4	5	$1.4x10^4M^{-1}$		
BSA	(+)-C	5	$0.9x10^4M^{-1}$	Polyphenol interactions through London dispersion forces with hydrophobic pockets and hydrophilic surface of protein	Soares et al. 2011
	malvidin-3-glucoside	5	$1.1x10^4M^{-1}$		
11S protein	chlorogenic acid	7	$4.5x10^3M^{-1}$	Hydrogen-bonding	Sastry & Rao 1990
	chlorogenic acid	5.5	$3.8x10^3M^{-1}$		

Interactions involved in protein-polyphenol interactions include hydrophobic interactions (specifically London forces), hydrogen binding, and electrostatic interactions. Generally, hydrophobic interactions are the driving force of protein-polyphenol

interactions, while hydrogen bonds tend to stabilize interactions (Murray et al. 1994; Papadopoulou et al. 2005; Xu and Chen 2011; Prigent et al. 2003; Jöbstl et al. 2006). For those polyphenols that are elongated and less hydrophobic, hydrogen bonding forces are more important than hydrophobic interactions, while compact hydrophobic polyphenols primarily interact with proteins through hydrophobic interactions (Hagerman et al. 1998). There have been less reports of involvement of electrostatic interactions in formation of protein-polyphenol complexes, however evidence exists that showssuch interactions may play a key role (Xu & Chen 2011).

Structural characteristics of polyphenols impact their affinity to bind protein. Binding appears to increase with polyphenol molecular weight, due to presence of aromatic rings that increase hydrophobicity and binding (Soares et al. 2007; Baxter et al. 1997; de Freitas et al. 2001). Large polyphenols may interact with proteins through multidentate association (Baxter et al. 1997; Charlton et al. 2002; de Freitas et al. 2001; Jöbstl et al. 2006). Alternatively, they may have low conformational mobility and thus low binding affinity for proteins (Soares et al. 2007; de Freitas et al. 2001; Baxter et al. 1997).

Substitution pattern on the polyphenol structure may also impact affinity for protein. For example, polyphenol galloylation increases binding interactions by initiating binding to either several sites on the same protein or in different protein molecules through hydrophobic effects and hydrogen binding (de Freitas et al. 2001; Baxter et al. 1997; Xiao et al. 2011; Xu & Chen 2011; Soares et al. 2007). Binding is also improved with increased hydroxylation. Specifically, hydroxyl groups on A and B rings of flavones and flavonols slightly enhanced interactions, hydroxyl groups on A rings of flavanones

significantly improved interactions (Xiao et al. 2011; Xu & Chen 2011), and presence of ortho phenolic hydroxyl groups appeared to increase interactions (de Freitas et al. 2001). Studies have also shown that structural characteristics of polyphenols that tend to decrease their affinity to protein include methylation, methoxylation, glycosylation, and hydrogenation of C2=C3 double bond (Xiao et al. 2011; Xu & Chen 2011; Papadopoulou et al. 2005).

Protein structure also influences formation of protein-polyphenol interactions. Studies show that proline residues on proteins are important for polyphenol binding, and proline residues followed by prolines are geometrically preferred polyphenol binding sites. Phenylalanine is another important amino acid that promotes interactions, although it is usually buried due to its hydrophobic nature. Histadine has been shown to be both important and unimportant in protein-polyphenol interactions (Yan et al. 1995; Naurato et al. 1999; Charlton et al 2002). Finally, arginine does not independently interact with polyphenols, however it appears to be associated with strengthening interactions (Baxter et al. 1997; Charlton et al. 2002; Soares et al. 2007).

As discussed, flavonoid interactions are typically within hydrophobic pockets and to hydrophobic residues of proteins (Papadopoulou et al. 2005; Soares et al. 2007). The size of the protein's hydrophobic cavity may thus dictate types of polyphenols the protein can interact with (Soares et al. 2007). Extent of protein secondary structure may therefore also dictate protein binding potential and potential sites. For example, globular proteins generally lack proline on their surface, whereas randomly coiled proteins have an abundance of proline residues on their surface and active for binding (de Freitas et al. 2001).

Stacking interactions between aromatic functions are also frequently involved in protein-polyphenol interactions (Baxter et al. 1997; Murray et al. 1994). The mechanism for binding between EGCG and proline-rich proteins has been investigated. The major conformation between proline-rich proteins and EGCG is the A ring over Pro5, D ring over Pro4, and B ring close to Arg (Charlton et al. 2002). Furthermore, changes in protein secondary structure due to protein-polyphenol interaction have been observed (Kanakis et al. 2011; Hasni et al. 2011).

Polyphenol-protein interactions are also dependent on several factors including polyphenol/protein ratio (Pascal et al. 2007), pH, temperature, and ionic strength in the context of solutions (Wang et al. 2010; von Staszewski et al. 2011, 2011b). Increasing both solution temperature and ionic strength promoted hydrophobic interactions between polyphenol and protein (Wang et al. 2010). Increasing ionic strength increased protein-polyphenol interactions by increasing electrostatic repulsions between protein/peptides, decreasing protein-protein interactions that occur at the protein's isoelectric point (pI).

1.6.2.1.1 Mechanisms and Factors Affecting Flavan-3-ol Protein Interactions

With specific regards to flavan-3-ols, interactions depend on structure of both flavan-3-ol and protein. For example, proteins with higher proline content favor polyphenol binding, and gallation of flavan-3-ols (EGC, EGCG, ECG, CG, GCG, GC) increases affinity of the compound to protein (Poncet-Legrand et al. 2007). Furthermore, size of the protein's hydrophobic cavity affects its interactions with flavan-3-ols: bovine serum albumin contains a large hydrophobic cavity favoring interactions with larger

gallated flavan-3-ols, whereas human salivary α-amylase has a multi-domain structure with small cavities optimal for binding small phenolics/polyphenols, such as gallic acid (Soares et al 2007).

Considering the common addition of milk to tea, it is no surprise that the interaction between tea flavan-3-ols and milk proteins has been explored. The primary interactions involved in complexation of flavan-3-ols and proteins were reported to be hydrophobic associations between flavan-3-ol rings and hydrophobic pockets of α- and β- casein and β-lactoglobulin. Hydrogen bonding interactions are believed to be involved in stabilization of these complexes. Binding of flavan-3-ols to caseins and β-lactoglobulin occurred primarily on the surface and within hydrophobic pockets of the protein, respectively. Increased polyphenol concentration decreased casein secondary structure leading to protein unfolding, but altered β-lactoglobulin secondary structure leading to stabilizion of protein structure under experimental conditions (Hasni et al. 2011; Kanakis et al. 2011).

1.6.2.2 Impact of Polyphenol-Protein Interactions on Food Quality

Effect of protein interactions with flavan-3-ols in complex foods has been a topic of several studies due, in part, to potential for the interactions to alter protein physical stability, product quality and also flavan-3-ol stability/activity. This has been most important with regards to beverage quality with specific respect to haze in juice, wine, and beer. Haze active proteins from cereals and fruit juices are active over a wide molecular weight range, and their ability to form complexes with polyphenols increases

by virtue of their proline content (Asano et al. 1982). Simple phenols and polyphenol monomers generally are not associated with haze (Asano et al. 1984). However larger flavan-3-ol dimers and polymers have haze activity that increases with polymerization (Mulkay & Jerumanis, 1983). Most haze is accentuated at lower temperatures that favor formation of hydrophobic and specific electrostatic interactions. These interactions are reversible and visually apparent haze can be reduced or eliminated when the beverage is warmed (Asano et al. 1982). Haze may also be prevented by prohibiting presence of oxygen from the system (Wall et al. 1996) and decreasing oxidation and polymerization of polyphenols. It may be reduced by adsorption of haze active proteins and polyphenols (Siebert and Lynn 1997a,b,c; 1998) and by utilization of enzymatic hydrolysis of haze active protein (de Clerck et al. 1969).

Impacts of polyphenol interactions with dairy protein on beverage nutrition and quality attributes have increasingly been studied due to common formulation of tea and cocoa with milk (Table 3). Potential for both interaction and oxidative processes in these systems creates a beverage matrix that does not favor delivery of flavan-3-ols. For example, addition of as little as 5% milk to tea was reported to decrease antioxidant activity of flavan-3-ol rich tea solutions as measured using both azinobis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS+) and voltammetry assays. This may have been due, in part, to binding and eventual precipitation of protein-polyphenol complexes thereby decreasing the amount of polyphenols available to react with oxidants in solution. A pronounced effect was observed in English breakfast tea models compared to Darjeeling and green teas, possibly due to presence of condensed flavan-3-ol polymers including theaflavins and thearubigins known to bind proteins to a

larger extent than flavan-3-ol monomers (Dubeau et al. 2010). The extent to which this effect was due to binding or a combination of binding and oxidative damage to polyphenols at elevated pH is not known. Conversely, the same study reported that tea antioxidant activity was increased in a lipid emulsion system, conceivably due to a synergistic effect between milk and tea polyphenols resulting from either facilitated deprotonation of polyphenols or creation of a physical protective barrier to lipid micelles.

Table 3: Impact of milk addition on antioxidant capacity of phenolic and polyphenolic rich beverages.

Beverage	Main phenolic class	Antioxidant Capacity	Reference
Coffee	Flavan-3-ols, Phenolic acids	Decreased; No effect	Niseteo; Dupas et al. 2006b
Tea	Flavan-3-ols	Decreased	Dubeau et al. 2010
Cocoa	Flavan-3-ols, Phenolic acids	Decreased	Belscak et al. 2009
Black tea	Monomeric and Polymeric Flavan-3-ols	Increased	Sharma et al. 2008

A study by Sharma et al. 2008 also produced results that initially appear conflicting. Formulation of milk (40%) to black tea decreased total free polyphenols and radical scavenging capacity measured using the 1,1-diphenyl-3-picrylhydrazyl (DDPH) model. This effect was presumably due to non-covalent interactions between polyphenol and protein. However, addition of milk, sugar, and their combination increased antioxidant activity in a β-carotene-linoleic acid model system. Antioxidant activity may have been more prevalent in the linoleic acid system compared to the DDPH model because many antioxidants are highly reactive to peroxyl radicals produced during lipid

peroxidation, but have low reactivity with the nitrogen radical in the DDPH model (Huang et al. 2005). These confounding effects make it difficult to fully interpret impact of flavan-3-ol-protein interactions.

Apparent masking of flavan-3-ol antioxidant capacity by milk proteins depends on structure of both protein and flavan-3-ols and follows typical binding parameters previously described. For example, Arts et al. 2002 concluded that a C3 gallate group increases extent of interaction between flavonoids and milk protein, while a 5'OH group decreases interaction. They also showed that proteins high in proline are likely to interact with hydroxyl groups of flavonoids. For example, the greatest antioxidant masking of EGCG (bearing a gallate group at the 3 position on C ring) and gallic acid occurred in combination with β-casein (a protein high in proline). In a similar study, precipitation of polyphenols by whey proteins from whey protein concentrate increased with green tea gallic acid equivalents, and antimicrobial activity decreased with protein concentration (von Staszewski et al. 2011b). This presumably indicates a decreased availability of bioactive flavan-3-ols for reactivity.

Additionally, salt concentration may have an impact on protein-polyphenol interactions. Whey protein concentrates with higher salt concentration (indicated by ash content) bound less effectively compared to those with lower salt concentration due to a 'screening' effect (von Staszewski et al. 2011b). Divalent cations including iron, copper, calcium, and magnesium may interact with electron dense phenolic rings of flavan-3-ols rendering them unavailable for participation in flavan-3-ol-protein interactions. Many studies have also demonstrated interactions between catechols and flavonoids with divalent metals (Kostyuk et al. 2004; Yamada et al. 2007; Vitali et al. 2008; Al-Numair

2009). Suggested mechanisms include chelation of salts by either two hydroxyl groups within catechols or by formation of salt bridges between multiple catechols (Yamada et al. 2007). These interactions have several effects on flavonoid stability and chromophore (Vitali et al. 2008; Al-Numair 2009; Mori et al. 2006; Lee et al. 2005) suggesting they may also modulate the ability of individual flavonoids to interact with proteins.

1.6.2.3 Impact of Polyphenol-Protein Interactions of on Polyphenol Bioavailability

Digestion and absorption of polyphenols have been previously discussed. Potential impact and interaction sites between protein and polyphenol are further outlined in Figure 5. It is clear that co-consumed proteins and polyphenols may interact throughout the digestive process. In fact potential impact of polyphenol-protein interactions on bioaccessibility and bioavailability of flavan-3-ols has been previously studied both *in vivo* and *in vitro.* Similar to effects on antioxidant activity and product quality, there have been many conflicting results (Tables 4 and 5, respectively).

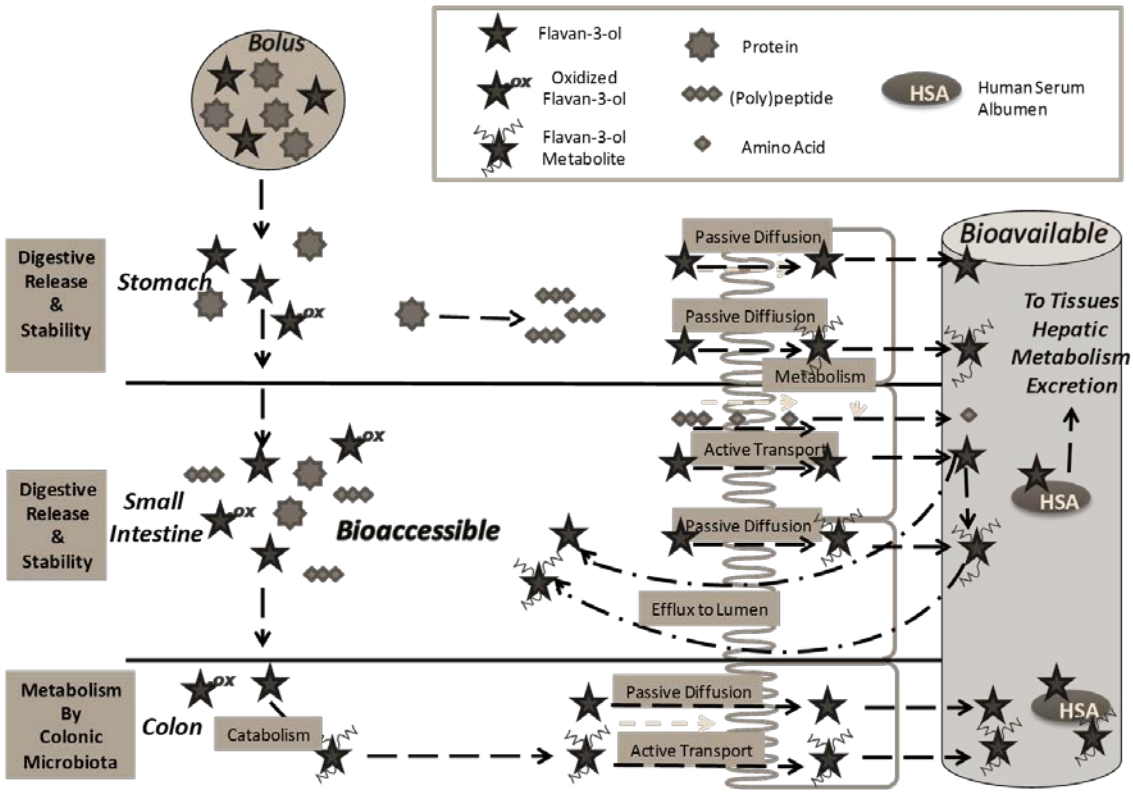


Figure 5: A schematic outlining bioavailability and bioaccessibility of proteins and polyphenols. Interactions between polyphenols and proteins/peptides may occur during digestion, altering polyphenol bioavailability.

Table 4: Impact of milk addition on *in vivo* bioavailability of polyphenols from foods and beverages in humans.

Food/Beverage	Polyphenol Analyzed	Flavan-3-ol Content	Milk Protein Content	Bioavailability	Boiled	Reference
Black tea	Total flavan-3-ol	196-203mg	2.3g	Yes; plasma AUC decreased from 1.14 to 0.95μmol/l over 3 hours	Yes	Reddy et al. 2005
Green tea, black tea	Total flavan-3-ol	0.9g, 0.3g in green and black tea, respectively	3.3g	No affect on plasma AUC over 8 hours	No	van het Hof et al. 1998
Black tea, green tea	Quercetin, kaempferol	~20mg flavonol in both types tea	3.9g	No affect on plasma AUC over 2 hours	Yes	Hollman et al. 2001
Black tea	Total flavan-3-ol	395umol flavan-3-ol	3.3g	No affect on plasma concentration (μmol) over 3 hours	Yes	Kyle et al. 2007
Cocoa (beverage)	C, (-) EC	2.0 - 2.7g polyphenol	2.45g	No affect on plasma concentration (μmol/l) over 6 hours	No	Keogh et al. 2007
Cocoa (beverage)	EC-glucuronide	64mg polyphenols	8.1g	No affect on plasma concentration (nmol/l) in after 2 hours	No	Roura et al. 2007
Cocoa (beverage)	(-)EC, flavan-3-ol	1.5mg/kg body weight (about 100mg)	260mg/kg (17.4g)	No affect on plasma C_{max} or AUC over 8 hours	No	Schramm et al. 2003
Cocoa (solid)	(-)EC	Not specified	6.5g	Yes; decreased plasma AUC by 46% over 4 hours when cocoa consumed with milk, and 69% when solid cocoa contained milk	No	Serafini et al. 2003
Cocoa (solid)	(-)EC	36mg	8.1g	No affect on C_{max} or T_{max} over 6 hours by addition of milk protein to solid cocoa formulation	No	Neilson et al. 2009
Blueberry fruit	Caffeic acid, ferulic acid	250mg polyphenol	6.5g	Yes; plasma AUC decreased by 49.7 and 19.8%, respectively, over 5 hours by consumption of blueberry with milk	No	Serafini et al. 2009

Several human studies have directly investigated the impact of milk on absorption of flavan-3-ols from tea (Table 4). Plasma flavan-3-ol concentration was higher in human subjects that ingested tea without milk compared to with milk (Reddy et al. 2005).

However, in contrast, bioavailability of flavan-3-ols from both green tea (van het Hof et al. 1998) and black tea (van het Hof et al. 1998; Kyle et al. 2007) were not affected by formulation with milk (17 and 25%) in healthy human subjects. Similarly, impact of green tea temperature on flavan-3-ol bioaccessibility from tea beverages formulated with and without milk was investigated with conflicting results (Table 5). Boiling green tea beverages enhanced *in vitro* bioaccessibility, and addition of milk to heated beverages decreased digestive recovery of flavan-3-ols to a greater extent compared to matching solutions at ambient temperature (Xie et al. 2012). This result may suggest increased protein-polyphenol interactions at elevated temperature. This effect may be due to increased exposure of surface hydrophobic residues on protein due to its denaturation, increasing its ability to interact with polyphenols through London forces. However, no apparent temperature-related trend for protein-polyphenol interactions is obvious when comparing across tea consumption and bioaccessbility studies (Tables 4 and 5).

Table 5: Impact of milk addition on *in vitro* bioaccessibility of polyphenols from foods and beverages.

Beverage	Polyphenol Analyzed	Flavan-3-ol Concentration	Milk Concentration	Affect Bioaccessibility	Boiled	Reference
Green tea extract	EGC, EC, EGCG, GCG, ECG	75 mg/mL	10 or 25% v/v	Yes	Yes, No	Xie et al. 2012
Green tea	EGC, EC, EGCG, ECG	60mg/mL	10, 20, 50% v/v	Yes	Yes	Green et al. 2007
Black tea	Total flavan-3-ols	1.8 mol/L	5.6-40%	No	Yes	van der Burg-Koorevaar et al. 2011

Similarly to studies with tea, bioavailability of flavan-3-ols from cocoa has also been studied with mixed findings (Table 4). Formulation of cocoa beverages with milk had no effect on plasma concentration of C, (-)-EC, and EC-glucuronide compared to controls (Keogh et al. 2007; Schramm et al. 2003; Roura et al. 2007). Conversely, *in vivo* bioavailability of (-)-EC from solid cocoa decreased to a significant extent in a study by Serafini et al. 2003, and modestly in another study (Neilson et al. 2009). Discrepancy in results between beverages and confections suggest that the physical state of the food matrix may impact protein-polyphenol binding during digestion, possibly due to affects on gastrointestinal motility and/or rate of flavan-3-ol solubilization in the gut (Neilson et al. 2009).

Interestingly, similar decreases in bioavailability have been observed in other foods/beverage phenolics (Table 4). Consumption of blueberries with milk resulted in a decrease in plasma concentrations of caffeic and ferulic acid *in vivo* (Serafini et al. 2009). While milk addition to coffee did not impact bioavailability of chlorogenic acid, addition of nondairy creamer slowed absorption (delay in T_{max}) of chlorogenic acid in humans (Reneuf et al. 2010). These data suggest that milk protein interactions with phenolics may have diverse impact on bioavailability measures.

1.6.2.4 Impact of Polyphenol-Protein Interactions on Biological Activities *in vivo*

Beyond absorption, ability of flavan-3-ol-protein interactions to modulate biological activities has been subject of several investigations (Table 6). Effect of co-formulation/consumption of protein on flavan-3-olantioxidant activity was also examined

in vivo. Addition of milk to both black and green tea did not decrease plasma antioxidant activity in humans (Leenan et al. 2000). Similarly chlorogenic acid in coffee was bound to dairy proteins without causing a significant effect on beverage antioxidant power. Incidentally, interactions were found to decrease during *in vitro* digestion suggesting release of phenolic compounds during normal digestion (Dupas et al. 2006a). However, addition of milk to black tea (10%) blunted the ability of tea to improve flow mediated dilation in humans and induce vasorelaxation in Wistar rat aortic rings (Lorenz et al 2007).

Table 6: Impact of milk addition to beverages on bioactivity.

Model	Polyphenol Dose/Concentration	Protein Dose/ Enzyme Concentration	Plasma Antioxidant Capacity	Flow Mediated Dilation	Enzyme Activity	Energy Gain	Reference
Human, *in vivo*, blueberry with milk	250mg	6.4g	No effect	-	-	-	Serafini et al. 2009
Human, *in vivo*, green, black tea with milk	640 and 140 mg for green and black tea, respectively	2.0g	No effect	-	-	-	Leenan et al. 2000
Human, *in vivo*, black tea with milk	450µmol	1.6g	-	Decrease	-	-	Lorenz et al. 2007
Human, *in vivo*, black tea with milk	450µmol	1.6g	-	Decrease	-	-	Lorenz et al. 2009
in vitro, green tea polyphenols, α-amylase, pepsin, trypsin, lipase,	0.05mg/mL	6-12ug/mL	-	-	Decrease	-	He et al. 2006
in vitro, oolong tea polyphenols, pancreatic lipase	2.5µg/mL	12.5U/mL	-	-	Decrease	-	Nakai et al. 2005
in vitro, flavonoids	0.05-2.3mM	3.2mg/mL (1,500U/mg) pepsin	-	-	Increase initial velocity	-	Tagliazucchi et al. 2005
in vitro, EGCG; α-amylase, trypsin, chymotrypsin	0.5mM	5, 50, and 50µg/mL, respectively	-	-	Decrease	-	Naz et al. 2011
Mouse, *in vivo*, EGCG	0.32%	-	-	-	-	Decrease	Grove et al. 2011
Rat, *in vivo*, tea flavan-3-ols	1%	-	-	-	-	Decrease	Unno et al. 2009
in vivo, green tea flavan-3-ols	199.3µmol	-	-	-	-	Decrease	Wang 2006b

Flavan-3-ol-protein interactions are believed alter macronutrient digestibility and availability (Table 6). Addition of green tea flavan-3-ols (mostly galloylated) at 1% w/w in diet significantly decreased body weight and increased fecal starch, protein, and lipid excretion in Wistar rats (Unno 2009). Similarly, decreased weight gain was observed in C57bl/6J mice fed a high fat diet formulated with 0.32% EGCG for 6 weeks compared to those fed matching diets void of EGCG. Furthermore, EGCG significantly increased fecal lipid content *in vivo* and competitively inhibited pancreatic lipase activity with 50% inhibition at 7.5µl/l *in vitro* (Grove et al. 2012). These data suggest that tea flavan-3-ols inhibit pancreatic lipase decreasing lipid absorption and concurrent weight gain. Not surprisingly, these interactions have stimulated interest in tea flavan-3-ols as anti-obesity agents.

Inhibition of specific digestive enzymes by flavan-3-ols has been studied extensively (Table 6). Percent inhibition of α-amylase, pepsin, trypsin, and lipase by 0.05 mg/mL tea derived flavan-3-ols was 61, 32, 38, and 54%, respectively (He et al. 2006). Similar results were reportedby Nakai et al. 2005 investigating effects of oolong tea polyphenols (monomeric and polymeric flavan-3-ols) on pancreatic lipase. Inhibition by polyphenols was attributed to presence of galloyl moieties and polymerization of flavan-3-ols, suggesting direct but non-specific binding as a primary mechanism. On the other hand, resveratrol, quercetin, EGCG, and flavan-3-ol increased initial velocity of pepsin activity during digestion of different substrates (Tagliazuchhi et al. 2005).

There is limited information on the mechanism behind polyphenol-digestive enzyme interactions. It appears conformational changes in digestive enzymes as a result of polyphenol binding can reduce their catalytic activity (Bandyopadhyay et al. 2012).

For example, binding of chlorogenic and caffeic acid to α-amylase altered the environment of the enzyme active site, thus causing inhibition of the enzyme (Raghavendra et al. 2007). Also, it appears that the high electron density of flavonols/flavones compared to flavan-3-ol/flavanones increases binding to digestive enzymes through stronger π-π interactions (Lo Piparo et al. 2008).

Interestingly, *in vitro* evidence suggests that the interactions of flavan-3-ols with digestive enzymes in the gut lumen may be modified by presence of food or salivary proteins. As described previously, proline-rich salivary proteins may also interact with EGCG, stabilizing it to digestion and also reducing its inhibitory effect on digestive enzymes (Naz et al. 2011). This experiment would seemingly support the notion that the astringent response derived from polyphenol interactions with salivary proteins would result in protection against anti-nutritional activities of polyphenols against digestive enzymes.

1.6.3 Milk Mineral-Milk Protein and –Polyphenol Interactions

1.6.3.1 Milk Mineral-Milk Protein Interactions

Milk and its products are used as ingredients in food applications for their nutritional and functional properties. Functional properties of milk and milk products including gelation, viscosity, emulsification, and foaming are largely attributed to type and amount of proteins present and interactions of proteins with milk components. Milk minerals constitute about 8-9 g/L of milk and are present as free ions and in complexes with macromolecules such as protein (West 1986; Lönnerdal 1988; Flynn 1992).

Solubility of milk protein-milk mineral complexes is important for protein functionality including properties such as emulsification, gelation and foam formation/stabilization (Swaisgood 1992). Additionally, these interactionas are believed to impact nutritional quality by modulating *in vivo* absorption and bioavailability of both proteins and minerals (Sanyal et al. 1992; Lönnerdalet al. 1994). Impact of milk mineral interactions with caseins and β-LG on protein structure and functionality has been subject of many reviews (Vegarud et al. 2000; McMahon & Oommen 2008; Gaucheron 2005; Augustin 2000).

Casein micelles in bovine milk are comprised of four proteins: α_{s1}- casein, α_{s2}-casein, β- casein, and κ-casein (Farrell et al. 2006). These caseins can interact with themselves, each other, and with minerals (ie. ionic calcium, calcium phosphate) to form a meta-stable supramolecular structure. Calcium bridging is an important interaction involved in formation of casein micelles in milk. Calcium phosphate nanoclusters appear to form interlocking sites between phosphoserine residues of multiple α_s- and β-caseins, termed “calcium bridges” (Holt et al. 1996) that provide structure-forming points for micelle formation (Holt et al. 2003). Cation binding capacity decreases with decreased phosphoserine content and is greatest for α_{s2}, followed by α_{s1}, β, and κ-casein (Holt, 1992). Following initial calcium cross-bridging, hydrophobic regions of caseins orient toward other proteins, forming linkages through non-covalent hydrophobic and electrostatic interactions. Further calcium bridging occurs through carboxylate and phosphoserine side chains of other caseins (Swaisgood 2003; Sood et al. 2002). The final casein micelle is a colloidal particle comprised of thousands of casein proteins and hundreds of calcium phosphate nanoclusters (de Kruif & Holt 2003).

Manipulation of minerals and mineral salts in dairy products results in altered protein functionality that may either improve or dimish the product's suitability for specific food applications. For example, milk with increased soluble calcium required increased heating time to coagulate (Kelly et al. 1982). In addition, increased calcium concentration promoted aggregation of rennetted micelles, promoting gel formation (Zoon et al. 1988; Udabage 1999). However, addition of calcium precipitated α_s or β-casein based emulsions presumably due to shielding of casein negative charges (Dalgleish 1987).

β-lactoglobulin is another milk protein that interacts with milk minerals. Simons et al. 2002 investigated the mechanism behind β-lactoglobulin-milk protein interactions through a series of tests including calcium-binding measurements, a combination of protein chemical modifications, and aggregation studies. Interestingly, they concluded that interactions occur due to screening of surface charges on β-lactoglobulin and subsequent decrease in electrostatic repulsion leading to aggregation, rather than due to formation of calcium bridges.

Many studies show that calcium enhances heat-induced aggregation of β-lactoglobulin (Anema & McKenna 1996; Changani et al. 1997; Sherwin & Foegeding 1997; Xiong et al. 1993). This property is useful for gelling applications. For example, microgels were developed by heating β-lactoglobulin witch calcium. For this application, amount of calcium required for gel formation decreased with decreasing pH, indicating that net charge density of the protein was crucial in microgel formation (Phan-Xuan et al. 2012). Conversely, this property causes concern in milk processing as it occurs optimally

during high temperature short time processing, therefore causing fouling of heat exchanges during milk processing.

1.6.3.2 Milk Mineral-Polyphenol Interactions

In addition to their interactions with proteins, many minerals in solution are well documented to interact with flavan-3-ols in both model and complex food systems. Mineral-flavan-3-ol interactions have been a subject of several investigations due to concerns surrounding their impact on mineral solubility and bioavailability. For example, the water-soluble fraction from olives contained polyphenol-bound magnesium (Yaşar & Güçe 2004). Similarly, concentration of intact phenolic compounds recovered from brewed herbal infusions was lower from tea infusions prepared with mineral water containing calcium and magenesium compared to with Milli-Q-quality water and tap water (Riehle et al. 2012), suggesting formation of mineral-flavan-3-ol complexes in polyphenol rich beverages prepared with mineral rich water. This complex formation has been associated with a phenomenon known as ‘tea cream’ that occurs in cooled black tea. Tea cream has been attributed to interaction between caffeine and phenolic compounds or self-association between phenolic compounds (Roberts et al. 1963; Jöbstl et al. 2005). Furthermore, addition of calcium to black tea enhanced self-association of caffeine, polyphenols, and therabugins and thus increased creaming, while addition of the chelating agent ethylendiaminetetraacetic acid (EDTA) decreased cream formation (Jöbstl et al. 2005; Smith 1989). Jackson and Lee 1988 discovered tannase, a polyphenol degrading enzyme, increased solubility of iron and calcium in green, oolong, black and

instant black tea, supporting the notion that break down of complex polyphenols would reduce formation of mineral-polyphenol complexes. Further, calcium and magnesium bioaccessibility from tea-biscuits was negatively correlated with polyphenol content, suggesting formation of insoluble polyphenol-mineral complexes that survive through simulated digestion.

Analysis of black tea stain formation on porcelain surface revealed a possible calcium-polyphenol interaction mechanism. Polymerization of polyphenols by oxidative reactions occurred during stain formation. Addition of calcium ions increased amount of stain and addition of EDTA was effective in removing black tea stains suggesting that calcium bridging between polyphenols may be involved in stain stability. Results from IR analysis (Yamada et al. 2007) suggest that calcium complexation with polyphenols occurs by either two hydroxyl groups within catechols or by calcium bridging formations between multiple catechols. From these results, it appears that minerals interact strongly with polyphenols and flavan-3-ols specifically through electrostatic interaction. There appears to be similarity between these interactions and those with proteins in that they have the ability to bind and form bridges between polyphenols. These similarities and potential for competitive interactions between milk protein, minerals and flavan-3-ols, it becomes important to consider presence and influence of milk minerals when investigating milk protein flavan-3-ol interactions. This has not been the case in many previous studies and has made interpretation of literature difficult. Additional insight in these interactions is needed in order to better understand the potential impact of milk protein-flavan-3-ol interactions in foods and beverages.

1.7 Conclusions and Research Objectives

In summary, a growing body of evidence supports the notion that polyphenol rich foods such as green tea can decrease risk of several chronic and degenerative diseases. Tea derived flavan-3-ols have been reported to have modifying activities against cardiovascular disease, cancer, diabetes, and cognitive impairment (Hooper et al. 2008; Miura et al 2000; Weseler et al. 2011; Wang et al. 2007; Hung et al. 2004; Zhong et al. 2001; Bonner et al. 2005; Ng et al. 2008; Kuriyama et al. 2006; Hu et al. 2007). However, bioavailability of flavan-3-ols is generally low. A key aspect of this poor bioavailability may be related to accessibility of flavan-3-ols to intestinal tissues for absorption (bioaccessibility). While instability of flavan-3-ols to digestive conditions has been previously documented to be a primary factor limiting bioavailabilitry (Yoshino et al. 1999; Zhu et al. 2002a; Green et al. 2007; Neilson et al. 2007; Ferruzzi et al. 2010), less is known regarding the extent to which protein-flavan-3-ol interactions may alter stability or bioaccessibility of flavan-3-ols. While a large portion of tea is currently formulated with milk, only a few studies have evaluated effects of milk proteins on green tea flavan-3-ol digestive stability and bioavailability. Interestingly, the physico-chemical nature of flavan-3-ol-protein interactions has been subject of several investigations that have yielded conflicting results regarding the nature and physiological consequences of such associations. A key component to these conflicting results may involve a more detailed consideration of the role of minerals in milk protein-flavan-3-ol interactions. Additional insight into nature and consequences of these interactions between milk protein, milk minerals and flavan-3-ols is required to leverage potential effects for optimal delivery of dietary flavan-3-ols.

The overall objective of this project is to develop a detailed understanding of the interaction between tea flavan-3-ols, milk proteins and milk minerals in model beverage systems. Specifically, we build on current knowledge of the nature of these interactions by assessing in a controlled fashion the comparative binding, stability and bioaccessibility of tea flavan-3-ols in model beverages. Our *central hypothesis* is that flavan-3-ol-protein interactions will increase digestive stability and bioaccessibility of flavan-3-ols through non-specific binding interactions with individual milk proteins in digestive environments. We further expect that these interactions may be altered by presence of milk minerals and that flavan-3-ol binding would modify protein digestibility and by extension release of flavan-3-ols in the digestive environment. Understanding how these interactions can alter digestive behavior of flavan-3-ols will lead to development of strategies that facilitate inclusion of flavan-3-ols in food and beverage system with improved stability and bioavailability.

To achieve our overall objectives, the following specific aims are proposed:

Specific Aim 1: Determine the impact of milk mineral and milk protein interactions on stability of flavan-3-ols in beverage systems and in the digestive environment. A three stage *in vitro* digestion model was utilized to assesss the impact of green tea formulation with milk proteins and a milk mineral buffer solution on digestive stability of flavan-3-ols. *Our working hypothesis was that the nature of flavan-3-ol-protein interaction will be dependent on both protein, presence of milk minerals and flavan-3-ol chemical composition and structure.* Stability would further depend on amount and type

of proteins in the food matrix. Binding with protein would lead to enhanced oxidative stability of flavan-3-ols through the digestive environment and thus higher digestive recovery.

Specific Aim 2: Characterize protein profile of individual milk protein digests resulting from *in vitro* digestion by pepsin and pancreatin. SDS-PAGE was utilized to evaluate protein degradation. *Our working hypothesis was that each milk protein would be rapidly degraded due to large amount of digestive enzymes used in this study.*

Specific Aim 3: Assess potential for binding between individual milk proteins digested to various extents and intact EGCG. Fluorescence spectroscopy was utilized to measure quenching of protein fluorescence by EGCG. *Our working hypothesis was that binding of EGCG to milk protein is altered by degradation of protein*. Binding data was used to formulate hypothesis related to *in vitro* digestion results.

CHAPTER 2. MATERIALS AND METHODS

2.1 Chemicals and Standards

Acetonitrile, acetic acid, methanol, water (Mallinckrodt-Baker, Phillipsburg NJ), and formic acid (Sigma-Aldrich St. Louis, MO) used in HPLC analysis were certified HPLC and ACS grade. Urea (U5378), uric acid (U2625), porcine mucin (M2378), α-amylase (A3176), pepsin (P7125), lipase (L3126), pancreatin (P1750), and bile salt (B8631) extract utilized for *in vitro* digestion were purchased from Sigma. KH_2PO_4 (VWR), K_2SO4 (Riedel-de Haën), potassium citrate, sodium citrate, KCl, $CaCO_3$, and $MgCO_3$ (Sigma-Aldrich) used in milk salt solution (JK) (Jenness & Koops, 1962) and Tris-HCl for fluorometry were purchased from Sigma-Aldrich. NaCl, HCl, and $NaHCO_3$ were purchased from Mallinckrodt (Phillipsburg, NJ). Powdered green tea extract (GT) was a gift from Nestlé PTC, Marysville, OH, USA. Acid precipitated casein was a gift from Kerry Group (Beloit, WI) (Figure D.1), and α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) were purchased from Sigma. Nonfat dry milk (NFDM) was Saco brand Mix 'n Drink. Coomassie Brilliant Blue R-250 for SDS-PAGE was purchased from Bio-Rad. Acrylamide, sodium thiosulfate, and tetramethylethylenediamine for SDS-PAGE were purchased from Sigma.

2.2 Preparation of Protein and Oral Phase Solutions for *in vitro* Digestion

Buffer (JK) for protein solutions was prepared following a procedure by Jenness & Koops, 1962. Specifically, KH_2PO_4, potassium citrate·H_2O, sodium citrate·$5H_2O$, K_2SO_4, $CaCO_3$, $MgCO_3$, and KCl, were dissolved in double distilled water (dd water) and pH adjusted to pH 6.3 with KOH to final concentrations of 1.58, 1.2, 2.12, 0.18, 1.32, 0.65, 0.3, 0.6, and 2.25 g/L KH_2PO_4, potassium citrate·H_2O, sodium citrate·$5H_2O$, K_2SO_4, $CaCl_2·2H_2O$, $MgCl_2·6H_2O$, K_2CO_3, KCl, and KOH respectively. Solutions containing individual milk proteins were formulated to mimic concentrations naturally occurring in milk. Sodium caseinate (S-CSN) was prepared in both dd water and JK by slowly stirring acid precipitated casein into solution for 2 hr, maintaining a pH of 6.3 by addition of 1.0 M NaOH. The final concentration of the solution was 35.6 mg/mL sodium caseinate. β-LG and α-LA solutions were prepared in JK similarly; however solutions were stirred for only 5 min. Final concentrations of β-LG and α-LA in JK were 3.5 and 1 mg/mL protein, respectively. NFDM was prepared in dd water (10% w/w). Oral phase solution was prepared by addition of KCl, Na_3PO_4, Na_2SO_4, NaCl, and $NaHCO_3$ for final concentrations of 242.78, 10.83, 8.03, 10.20, and 40.33 mmol/L, respectively.

2.3 Model Tea Beverage Preparation

GT was dissolved in dd water (3 mg/mL). The flavan-3-ol composition of the tea powder is described in Table 7. Test solutions with tea were designed to deliver 0.60 mg/ml of total tea flavan-3-ols (0.18, 0.032, 0.33 and 0.054 mg/mL of EGC, EGCG, EC and ECG respectively). The flavan-3-ol content was equivalent to that found in ~0.5-1 cup of tea (Bahgwat et al. 2011). Powdered tea extract solutions formulated with 10, 20,

and 40% (v/v) of aforementioned protein solutions matching tea powder free controls (dd water, JK) were prepared.

Table 7: Flavan-3-ol content in green tea powder.

Flavan-3-ol	mg/g tea extract powder
EGC	60.31 ± 1.29
EC	10.86 ± 0.77
EGCG	110.05 ± 2.35
ECG	17.85 ± 0.33

2.4 Three Stage *in vitro* Digestion of Model Tea Beverages

Aliquots of freshly prepared tea beverages were subjected to simulated oral, gastric and small intestinal digestion in sequence as previously described by Thakker et al. 2007 modified by Kean et al 2011. Briefly, 6 mL oral phase solution was added to the formulated beverages (20 mL) described above. The reaction tube was vortexed briefly to ensure mixing, blanketed with nitrogen, and incubated in a covered shaking water bath (37°C, 85 opm, 10 min). Samples were then diluted to 30 mL with saline. The gastric phase was initiated by addition of 2 mL porcine pepsin solution (20 mg/mL in 0.1 M HCl) and adjustment of the pH to 3.0 ± 0.1 with 1.0 M HCl. After addition of saline for 40 mL total solution, samples were blanketed with nitrogen and incubated in a covered shaking water bath (37°C, 85 opm, 1 hr). The small intestinal phase was initiated by addition of 1.0 M $NaHCO_3$ to raise the pH of gastric digesta (GD) to 6.5 ± 0.1. A cocktail of small intestinal enzymes was added to the solution (2 mL, 30 mg/mL pancreatin, 15 mg/mL

lipase in 0.1 M $NaHCO_3$) as well as porcine bile salts (3 mL, 120 mg/mL bile extract in 0.1 M $NaHCO_3$). The pH was then readjusted to 6.5 ± 0.1 with 1.0 M HCl and saline was added for 50 mL total solution. Samples were then blanketed with nitrogen and incubated in a covered shaking water bath (37°C, 85 opm, 2 hrs). After completion of the small intestinal phase, samples were centrifuged (10,400 x g, 4°C, 1 hr). Aliquots of undigested beverage starting material (SM), GD, and centrifuged aqueous small intestinal digesta (DG) were collected and acidified with 2% aqueous acetic acid (1:1) and stored frozen at -80°C under a blanket of nitrogen until analysis by HPLC.

2.5 Flavan-3-ol Analysis by HPLC

Thawed aliquots of SM tea-protein beverages were extracted prior to analysis following an enzyme-assisted extraction previously reported (Ferruzzi & Green 2006). Aliquots from DG and extracted SM were diluted 1:1 with 2% acetic acid in dd water and centrifuged at 14,000 x g for 5 minutes. Supernatants from both SM and DG were collected and filtered through a 0.45 μm PTFE filter prior to analysis. 10 μL of resolubilized sample was injected on an HP 1050 HPLC system equipped with a 12 channel CoulArray Multi-channel ECD Array system (ESA Inc). Separation was achieved according to Peters et al. (2010) with minor modification. A Waters Xterra RP-C18 column (3.9 mm id x 100 mm, 3.5 μM particle size) preceded by a guard column packed with the same stationary phase was used to separate flavan-3-ols under gradient conditions at a flow rate of 0.8 mL/min using a binary mobile phase of ddH_2O, acetonitrile, formic acid (91.9:8:0.1) in reservoir A and ddH_2O, acetonitrile, methanol, triflouroacetic acid (49.9:47:3:0.1) in reservoir B. Initial conditions of 90:10 A/B was

followed by a linear gradient to 50:50 A/B (0-5 min), 10:90 A/B (5-7 min), 10:90 A/B (7-10 min), 90:10 A/B (10-12 min), and 90:10 A/B (12-15 min). 12-channel ECD was performed with cell potentials at -100, 120, 180, 240, 300, 360, 420, 480, 540, 600, 660, and 720 mV. Flavan-3-ols were quantified by summing responses obtained at 120, 180, 240, and 300 mV (channels 2-5).

2.6 Assessment of Protein-Flavan-3-ol Interactions Through *in vitro* Digestion

2.6.1 *In vitro* Digestion of S-CSN, β-LG, and α-LA for Assessment by SDS-PAGE

S-CSN, β-LG, and α-LA were digested *in vitro* for analysis by SDS-PAGE. To obtain simple digests for clean SDS-PAGE analysis, digestions were carried out such that only target protein, JK buffer, saline, pepsin, and pancreatin were present in final digests. Digestions were completed in quadruplicate representing gastric times 0 and 1 hour, and duodenal times 15, 30, 60, and 120 mins. Beverages (5.6 mL) containing protein (35.6 mg/mL acid casein, 1 mg/mL α-LA, or 3.5 mg/mL β-LG in JK) were mixed with 0.9% saline for 8.4 mL total solution. The pH was adjusted to 3.0 ± 0.1 using 1.0 M HCl followed by addition of pepsin solution (0.56 mL, 20 mg/mL) to simulate the gastric phase. Gastric time 0 (G0) samples were frozen (-20°C), and freeze-dried for analysis. Remaining samples were standardized to 11 mL, blanketed with nitrogen gas, parafilmed, and incubated in a water bath (85 opm, 1 hr, 37°C). After incubation, gastric time 1 hour (G1) samples were frozen (-20°C) and freeze-dried for analysis. Remaining samples were adjusted to pH 6.5 ± 0.1 using 1.0 M $NaHCO_3$. Pancreatin solution (0.56 mL, 30 mg/mL) was added to simulate the duodenal phase, the pH was readjusted to 6.5, saline was added for 14 mL total solution, and samples were covered with nitrogen, parafilmed, and

incubated in a water bath (85 opm, 37°C) for 15, 30, 60, and 120 minutes. Duodenal samples (D15, D30, D60, D120) were collected, frozen (-80°C), and freeze-dried for analysis.

2.6.2 SDS-PAGE Analysis for Characterization of Milk Protein Digests

Raw material (α-LA, β-LG, S-CSN, pancreatin and pepsin) and freeze-dried digests were characterized by SDS-PAGE using a Bio-Rad Mini-PROTEAN Tetra cell (BioRad Laboratories, California, USA) using hand-casted gels (Gravel 2002). 10-20% polyacrylamide gels were utilized for separation of proteins from raw materials and S-CSN and β-LG digests. A 10% polyacrylamide gel was utilized for separation of protein from α-LA digest. Freeze-dried samples from *in vitro* digestion of S-CSN, α-LA, and β-LG were resolubilized in 10 mL, 280 μL, and 1 mL, respectively, of Tris-HCl (10 mM, pH 7.4) for about 8 mg/mL protein total in sample. Samples were mixed 1:1 with Bio-Rad 2x Laemmli buffer and boiled for 5 minutes before application onto gel. The gels were stained using 2% Coommassie Blue solution and destained (Gravel 2002). Molecular weights were estimated using a Bio-Rad broad range molecular weight marker calibration kit (6,500 to 200,000 Da) for raw materials and S-CSN and β-LG digests, and a Precision Plus Protein™ Kaleidoscope™ Standards calibration kit (10,00 to 250,000 Da) for α-LA digest.

2.6.3 Evaluation of Milk Protein-EGCG Interactions through Fluorescence Spectroscopy

The i*n vitro* digestion procedure used prior to analysis by SDS-PAGE (outlined in section 2.6.1) was used with minor modification to digest S-CSN for fluorescence analysis to determine extent of protein Trp fluorescence quenched by EGCG at various points of digestion. S-CSN (0.023 g/L) was prepared in Tris-HCl (10 mM, pH 7.4) and pH adjusted with NaOH to pH 6.3. Final digesta contained 0.0115 mg/mL S-CSN, 0.005 mg/mL pepsin, and 0.006 mg/mL pancreatin and had final pH of 3.0 or 6.5 for gastric and duodenal digesta, respectively. According to preliminary results (See Appendix B), aforementioned concentrations are appropriate to ensure S-CSN fluorescence is within linear range of fluorometerand digestive enzyme Trp fluorescence interferes minimally with S-CSN fluorescence. Gastric time zero (G0) samples were prepared by addition of 1 mL pepsin solution (0.07 mg/mL in 0.1 M HCl) and 1 mL pancreatin solution (0.084 mg/mL in 0.1 M $NaHCO_3$) to 7 mL S-CSN solution. G0 samples were standardized to 14 mL with Tris-HCl pH 3.0, pH adjusted to 3.0, and frozen (-20°C). For remaining samples, S-CSN (7 mL) was pH adjusted to 3.0 ± 0.1 by addition of 1.0 M HCl and made up to 8mL with saline. Subsequently, the gastric phase was initiated by addition of 1 mL pepsin solution (0.07 mg/mL in 0.1M HCl) and samples were blanketed with nitrogen gas and incubated in a shaking water bath (85 opm, 37°C, 1 hr).Pancreatin (1 mL, 0.084 mg/mL in 0.1 M $NaHCO_3$) was then added to gastric time one hour (G1) samples which were made up to 14 mL with Tris-HCl pH 3.0, pH adjusted to 3.0, and frozen (-20°C). The duodenal phase was initiated in remaining solutions by pH adjustment to 6.5 ± 0.1 and

addition of 1mL pancreatin (0.084 mg/mL in 0.1 M $NaHCO_3$). Samples were then readjusted to pH 6.5 ± 0.1, made up to 12 mL with saline, blanketed with nitrogen gas and incubated in a shaking water bath (85 opm, 37°C). Digestions were stopped at either 15, 30, 60, or 120 mins (D15, D30, D60, D120), made up to 14 mL with Tris-HCl pH 6.5, pH adjusted to 6.5, and frozen (-20°C).

Additional experiments using S-CSN as a milk protein model were executed to determine (1) extent of binding between EGCG and digestive enzymes and (2) S-CSN-EGCG binding effects due to digestive pH. For both experiments, the digestion procedure outlined in the previous paragraph was mimicked with minor changes (Table 8). Extent of binding between EGCG and digestive enzymes was determined by incubating pepsin and pancreatin at pH 3.0 and/or 6.5 without S-CSN prior to fluorometry. To determine S-CSN-EGCG binding effects due to digestive pH, S-CSN was incubated at pH 3.0 and/or 6.5 without presence of digestive enzymes prior to fluorometric analysis.

Table 8: Experimental conditions of digestions preceding fluorescence spectroscopy used to determine (1) extent of binding between EGCG and digestive enzymes, (2) S-CSN-EGCG binding effects due to digestive pH and (3) combined effect of protein digestion, pH, and presence of enzymes in S-CSN digesta on fluorescence quenching of protein Trp residues by EGCG.

Effect	S-CSN	Pepsin and Pancreatin	pH of gastric digesta solution for fluorometry	pH of dudoenal digesta solution for fluorometry
(1) EGCG-enzyme	No	Yes	3.0	6.5
(2) pH	Yes	No	3.0	6.5
(3) Full system	Yes	Yes	3.0	6.5

Fluorometric experiments were carried out on a Perkin-Elmer LS55 Spectrometer (Woodbridge, ON, Canada). Method development was initiated following a method by Kanakis et al. 2011.

An EGCG stock solution (12 µM in Tris-HCl (10 mM, pH 3.0 or 6.5)) was prepared and kept on ice for analysis. EGCG was stable under these conditions with 98% recovery over 6 h. S-CSN digested to various extents *in vitro* wasmixed 1:1 with EGCG by inverting solution 10 times. EGCG concentration of each solution was about 6 µM, and protein concentration of S-CSN was 0.0115 g/L. Pepsin and pancreatin were also present from background digestion enzyme mixes at 0.005 and 0.006 mg/mL, respectively, therefore, blanks containing milk protein and background digestive enzyme mix mixed 1:1 with Tris-HCl (10 mM, pH 3.0 or 6.5) replacing EGCG were prepared. Fluorescence spectra were obtained using λ_{exc}=280nm and λ_{emi} from 290-500nm. Intensity at 350 nm (Trp) was recorded.

Percentage fluorescence quenched was calculated using the following formula:

$$\%F_q=[(F_0-F)/F_0]100$$

Where: F_0= initial fluorescence intensity (intensity of blank)

F= fluorescence intensity in presence of quenching agent

Preliminary tests showed that EGCG mixed 1:1 with Tris-HCl (buffer) fluoresced less than just Tris-HCl (Table C.1). In addition, it appeared EGCG quenched digestive enzyme fluorescence. Due to its low impact on fluorescence and ability to quench some enzyme fluorescence, EGCG fluorescence was not corrected for in calculations for percent quenching of Trp residues by EGCG.

2.7 Data Analysis

Results calculated from LC-ECD data include both *relative* and *absolute bioaccessibilities* of EGC, EC, EGCG, and ECG from each beverage. Bioaccessibility is a measure of the digestive release and stability of a compound in the gut and is required for the compound's subsequent absorption. *Relative bioaccessibility* is defined as the percent of flavan-3-ol recovered in final digesta relative to that in starting material. *Absolute bioaccessibility* is the amount of flavan-3-ol available for absorption defined as the relative bioaccessibility multiplied by grams of starting material and provides information on impact of changes in digestive recoveries of each flavan-3-ol relative to their abundance in GT. Percentage fluorescence quenched ($\%F_q$) is the ratio of amount of protein fluorescence lost due to addition of quencher (EGCG) compared to that of

original protein fluorescence. Increased $\%F_q$ suggests increased quencher binding to Trp residues. SDS-PAGE was used to separate peptides resulting from digestion of individual proteins. Protein bands were compared to molecular weight ladder (6.5-200 kDa for S-CSN and β-LG digests, 10-250 kDa for α-LA digests). Fading of bands and appearance of lower molecular weight bands with increasing extent of digestion suggest degradation of protein or peptide to smaller peptides. *In vitro* digestion and fluorometry data represent mean +/- standard error of the mean of four individual samples. Analysis by one-way ANOVA was performed using SAS 9.3 (SAS Institute, Cary, NC) for each variable and control. Significant differences between treatments were evaluated by a Bonferroni method ($\alpha < 0.05$).

CHAPTER 3. RESULTS

Consequence of milk addition on bioaccessibility and bioavailability of green tea flavan-3-ols has been previously investigated using both *in vivo* and *in vitro* models. Although results are conflicting between botanical matrices and approaches, there appears to be consensus that there is potential for milk protein interaction to alter bioaccessibility of tea flavan-3-ols in the gastrointestinal (GI) tract (Xie et al. 2012; Green et al. 2007; van der Burg-Koorevaar et al. 2011) (Section 1.6.2.3, Table 5). However, extent of this effect and putative involvement of individual milk proteins in such interactions remain unclear. Additionally, many previous studies did not addresss the role of milk minerals to modulate flavan-3-ol digestive stability or capacity for protein binding. The purpose of this study was therefore to investigate extent to which tea derived polyphenols (the flavan-3-ols) interact with specific milk proteins (α-LA, β-LG, S-CSN), at relevant beverage concentrations and to determine the impact of these interactions on digestive stability and bioaccessibility of green tea flavan-3-ols in an *in vitro* three-stage gastrointestinal model.

There was no significant difference ($p>0.05$) in flavan-3-ol bioaccessibility between green tea beverages formulated with 10, 20, and 40% of test material (Figures A.1, A.2, A.3, and A.4 in Appendix). Therefore, results and discussion will be based on relative and absolute bioaccessibility of green tea beverages formulated with 40% test material.

As mentioned above, absolute bioaccessibility will be discussed to provide information on the impact of changes in digestive recoveries of each flavan-3-ol relative to their abundance in GT.

3.1 Starting Flavan-3-ol Profile of Green Tea Extract Beverages

Starting levels (SM) of total flavan-3-ols ranged from 42.16 to 57.52 μmol/digestion reaction (50 mL of model tea beverage) (Table 9). Beverages formulated with NFDM had significantly lower ($p<0.05$) total flavan-3-ol level compared to beverages formulated with JK control and α-LA, β-LG, and S-CSN solubilized in JK: 42.16±0.36 μmol of total flavan-3-ols per reaction compared to 52.18±0.20, 52.21±0.68, 49.05±0.77, and 57.52±1.90 μmol of total flavan-3-ols per reaction respectively. In addition, SM level of total flavan-3-ols in beverages formulated with S-CSN in buffer (S-CSN/JK) was significantly higher ($p<0.05$) compared to S-CSN in dd water (S-CSN/water) and dd water controls with 57.52±1.90 compared to 48.56±0.61 and 46.33±2.19 μmol/digestion reaction respectively. No further differences in SM total flavan-3-ols levels were observed.

Table 9: Total flavan-3-ols in undigested model beverages (starting material-SM) and digested model beverages (digesta-DG).Presence of different letters in same column indicates significantly lower flavan-3-ol content between formulations.

Treatment	Total Flavan-3-ols in SM (μmol/digestion reaction)	Total Flavan-3-ols in DG (μmol/digestion reaction)
NFDM	42.16 ± 0.36[a,b]	15.32 ± 0.38[a]
JK Control	52.18 ± 0.20[a,c]	16.86 ± 0.66[b]
α-LA	52.21 ± 0.68[a]	13.90 ± 0.74[a]
β-LG	49.05 ± 0.77[a]	14.48 ± 0.28[a]
S-CSN in JK	57.52 ± 1.90[b]	5.91 ± 0.45[b]
dd Water Control	48.56 ± 0.61[a,c]	8.64 ± 0.50[b]
S-CSN in Water	46.33 ± 2.19[c]	5.42 ± 0.26[a]

Table 10: Bioaccessibility (% and μg/g) of (a) EGC, (b) EC, (c) EGCG, and (d) ECG from green tea beverages formulated with non-fat dry milk, buffer, water; α-LA, β-LG and S-CSN/buffer; and S-CSN/water. Presence of different letters in same column indicates significantly lower flavan-3-ol content between formulations.

(a)

Treatment	μmol/digestion reaction (SM)	μmol/digestion reaction (DG)	Relative Bioaccessibility (%)	Absolute Bioaccessibility (μmol/g)
Non-Fat Dry Milk	13.54 ± 0.09^{a}	9.72 ± 0.25^{a}	71.9 ± 1.4^{e}	141.6 ± 2.7^{c}
JK Control	15.92 ± 0.08^{a}	7.90 ± 0.64^{a}	49.9 ± 4.7^{a}	98.3 ± 9.2^{a}
α-LA	15.71 ± 0.26^{a}	7.12 ± 0.47^{a}	$45.5 \pm 2.8^{a,b}$	89.6 ± 5.6^{a}
β-LG	15.49 ± 0.38^{a}	8.44 ± 0.25^{a}	$54.7 \pm 0.3^{a,e}$	$107.6 \pm 0.6^{a,c}$
S-CSN/JK	15.67 ± 0.76^{a}	$1.40 \pm 0.42^{b,c}$	9.1 ± 2.4^{c}	17.8 ± 4.7^{b}
Water Control	15.01 ± 0.23^{a}	4.08 ± 0.49^{b}	$30.2 \pm 0.9^{b,c}$	59.4 ± 1.8^{b}
S-CSN/Water	11.35 ± 1.55^{b}	0.86 ± 0.24^{c}	6.8 ± 1.0^{d}	13.5 ± 2.0^{b}

(b)

Treatment	μmol/digestion reaction (SM)	μmol/digestion reaction (DG)	Relative Bioaccessibility (%)	Absolute Bioaccessibility (μmol/g)
Non-Fat Dry Milk	3.70 ± 0.03^{c}	2.53 ± 0.09^{b}	68.7 ± 2.9^{c}	25.7 ± 5.7^{b}
JK Control	$4.53 \pm 0.02^{a,b}$	3.73 ± 0.06^{a}	82.6 ± 1.4^{a}	30.9 ± 0.5^{a}
α-LA	4.21 ± 0.21^{a}	3.34 ± 0.15^{c}	79.7 ± 2.0^{a}	29.8 ± 3.9^{a}
β-LG	$4.39 \pm 0.11^{a,b}$	$2.73 \pm 0.08^{b,c}$	$62.8 \pm 2.9^{b,c}$	23.5 ± 5.6^{b}
S-CSN/JK	4.76 ± 0.04^{b}	$2.74 \pm 0.01^{b,c}$	58.1 ± 0.6^{b}	21.7 ± 1.2^{b}
Water Control	$4.15 \pm 0.07^{a,c}$	2.55 ± 0.03^{b}	$61.3 \pm 1.1^{b,c}$	$22.9 \pm 2.3^{b,c}$
S-CSN/Water	$4.46 \pm 0.12^{a,b}$	3.00 ± 0.07^{c}	$67.9 \pm 0.4^{b,c}$	25.4 ± 0.8^{c}

(c)

Treatment	μmol/digestion reaction (SM)	μmol/digestion reaction (DG)	Relative Bioaccessibility (%)	Absolute Bioaccessibility (μmol/g)
Non-Fat Dry Milk	20.33 ± 0.34^{c}	2.62 ± 0.27^{c}	13.1 ± 1.2^{c}	$31.4 \pm 2.4^{a,b}$
JK Control	26.42 ± 0.17^{a}	4.41 ± 0.13^{a}	16.9 ± 0.5^{a}	40.5 ± 1.2^{a}
α-LA	$26.64 \pm 0.57^{a,b}$	2.89 ± 0.54^{c}	$10.9 \pm 1.8^{b,c}$	26.2 ± 3.5^{b}
β-LG	$24.07 \pm 0.65^{a,c}$	2.81 ± 0.09^{c}	11.9 ± 0.6^{c}	28.6 ± 1.1^{b}
S-CSN/JK	31.00 ± 1.69^{b}	$1.39 \pm 0.16^{b,c}$	4.7 ± 0.6^{d}	11.3 ± 1.2^{c}
Water Control	$24.13 \pm 0.55^{a,c}$	$1.63 \pm 0.08^{b,c}$	$7.1 \pm 0.2^{b,d}$	16.9 ± 0.5^{b}
S-CSN/Water	24.78 ± 1.52^{a}	1.14 ± 0.05^{b}	4.9 ± 0.1^{d}	11.7 ± 0.2^{b}

(d)

Treatment	μmol/digestion reaction (SM)	μmol/digestion reaction (DG)	Relative Bioaccessibility (%)	Absolute Bioaccessibility (μmol/g)
Non-Fat Dry Milk	4.59 ± 0.08^{c}	0.46 ± 0.05^{b}	10.9 ± 1.1^{b}	4.4 ± 2.1^{b}
JK Control	$5.30 \pm 0.05^{a,b,c}$	0.81 ± 0.02^{a}	16.1 ± 0.4^{a}	6.5 ± 0.2^{a}
α-LA	$5.64 \pm 0.14^{a,c}$	0.55 ± 0.08^{b}	$10.4 \pm 1.1^{b,c}$	4.2 ± 2.2^{b}
β-LG	$5.10 \pm 0.14^{a,b}$	0.50 ± 0.02^{b}	10.7 ± 0.6^{b}	4.3 ± 1.2^{b}
S-CSN/JK	6.09 ± 0.39^{b}	0.16 ± 0.02^{b}	7.1 ± 0.6^{c}	2.9 ± 1.2^{b}
Water Control	$5.27 \pm 0.12^{a,b,c}$	0.39 ± 0.01^{b}	$8.1 \pm 0.2^{b,c}$	$3.3 \pm 0.4^{b,c}$
S-CSN/Water	$5.75 \pm 0.27^{a,b}$	0.42 ± 0.00^{b}	$8.2 \pm 0.4^{b,c}$	$3.3 \pm 0.7^{b,c}$

Individual tea flavan-3-ols in SM including EGC, EC, EGCG, and ECG ranged from 3.7 to 31.0 μmol/digestion reaction (Figures 6 and 8, Table 10). No significant difference in individual SM flavan-3-ol level was found between dd water and buffer formulations. For individual flavan-3-ols in NFDM beverages, EC (3.70±0.03 μmol/digestion reaction) was significantly lower ($p<0.05$) compared to all treatments except dd water; EGCG (20.33±0.34 μmol/digestion reaction) was lower compared to JK buffer ($p<0.05$) and beverages formulated with S-CSN and α-LA ($p<0.05$); and ECG (4.59±0.08 μmol/digestion reaction) was lower compared to beverages formulated with S-CSN and β-LG ($p<0.05$). In addition, SM levels of EGCG in model beverages formulated with S-CSN/JK (31.00±1.69 μmol/digestion reaction) were significantly higher ($p<0.05$) compared to all treatments except α-LA. Levels of individual flavan-3-ols in beverages formulated with JK buffer, α-LA, and β-LG were not significantly different from each other.

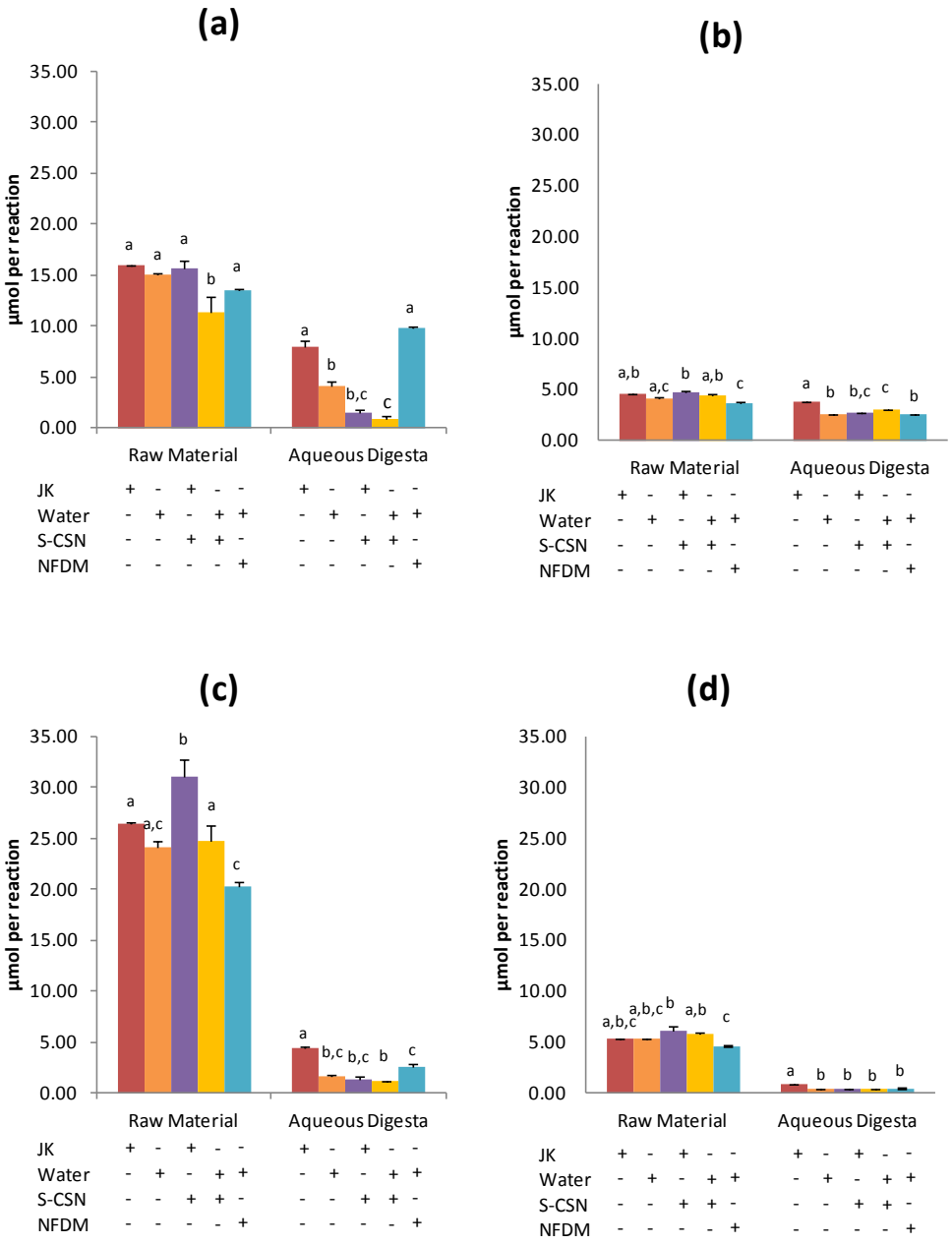


Figure 6: Levels of (a) EGC, (b) EC, (c) EGCG, and (d) ECG in starting material and aqueous digesta of green tea beverages formulated with sodium caseinate in JK buffer or dd water and dd water and JK buffer controls. Presence of different letters within raw material or aqueous digesta data indicates significantly different flavan-3-ol content between formulations.

3.2 Green Tea Flavan-3-ol Bioaccessibility in Milk Mineral Buffer (JK)

Initial flavan-3-ol levels in model beverage systems (SM) following simulated oral, gastric and small intestinal digestion were assessed (Figures 6 and 8, Table 10) to determine the impact of milk minerals on flavan-3-ol bioaccessibility. Initial assessment focused on the impact of JK buffer relative to dd water on tea flavan-3-ol *in vitro* digestive stability/bioaccessibility. It appears that presence of milk minerals from JK buffer significantly increased digestive stability of flavan-3-ols relative to dd water formulation ($p<0.05$).

Total flavan-3-ols levels in JK buffer control final digesta (DG) (16.86±0.66 μmol/digestion reaction) was significantly higher ($p<0.05$) than in dd water control DG (8.64±0.50 μmol/digestion reaction) (Figure 6, Table 9). Beverages formulated with JK buffer had significantly higher ($p<0.05$) DG levels of EGC, EC, EGCG, and ECG compared to dd water control beverage with 7.90±0.64, 3.73±0.06, 4.41±0.13 and 0.81±0.02 μmol/digestion reaction compared to 4.08±0.49, 4.15±0.07, 1.63±0.08, and 0.39±0.01 μmol/digestion reaction respectively (Table 10). These levels represent a significant increase ($p<0.05$) in bioaccessibility of all tea flavan-3-ols in beverages formulated with JK buffer compared to those formulated with dd water from 30.2±0.9, 61.3±1.1, 7.1±0.2, and 8.1±0.2% to 49.9±4.7, 82.6±1.4, 16.9±0.5, and 16.1±0.4% for EGC, EC, EGCG, and ECG, respectively (Figure 7, Table 10). These values represent increases in absolute bioaccessibilities from 59.4±1.8, 22.9±2.3, 16.9±0.5, and 3.3±0.4 μmol/g for dd water controls to 98.3±9.2, 30.9±0.5, 40.5±1.2, and 6.5±0.2 μmol/g for buffer controls, respectively.

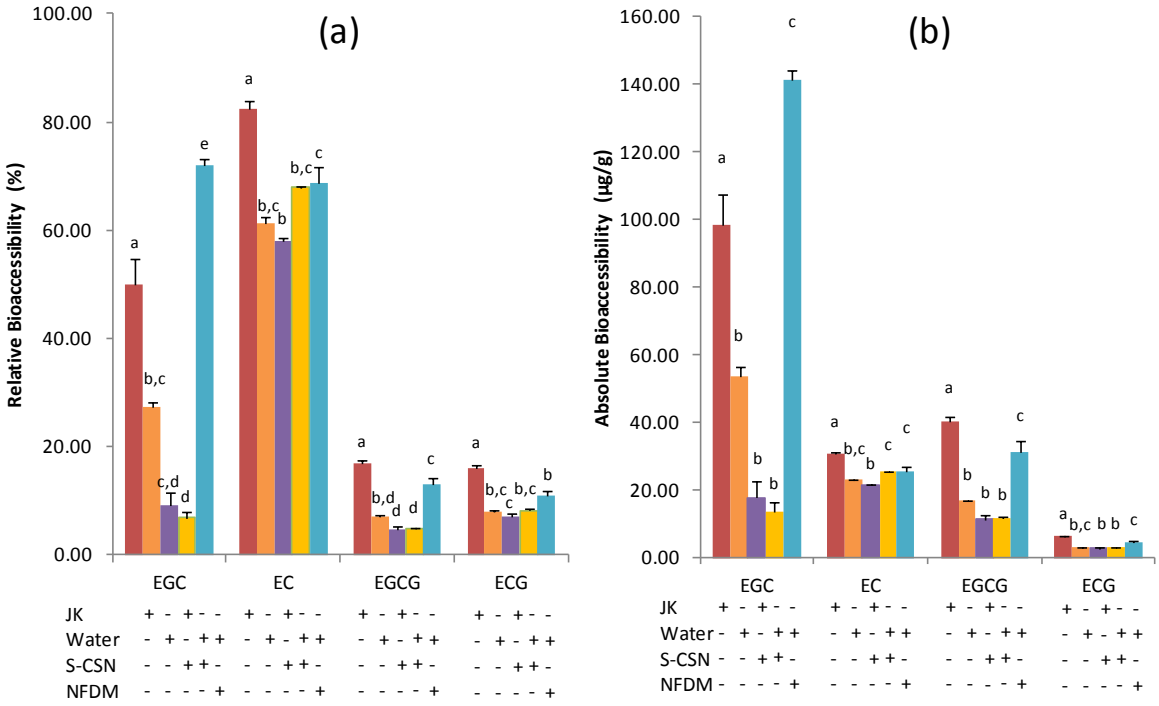


Figure 7: (a) Relative (%) and (b) absolute (µg/g) bioaccesibility of flavan-3-ols from green tea beverages formulated with sodium caseinate in dd water or buffer, and dd water and buffer controls.Presence of different letters indicates significantly different flavan-3-ol recovery between formulations.

3.3 Green Tea Flavan-3-ol Bioaccessibility in Non-Fat Dry Milk (NFDM) Formulations

Green tea beverages were formulated with NFDM to determine effects of the full milk matrix on flavan-3-ol digestive stability and bioaccessibility. Bioaccessibility of flavan-3-ols from beverages formulated with NFDM was compared with dd water control to parallel past studies concerning bioaccessibility of tea flavan-3-ols from beverages formulated with milk/creamer compared to dd water controls. NFDM is obtained by removal of water only (21CFR131.125) and thus reconstituted NFDM used in this study

contained both milk proteins and milk minerals in the same relative proportions found in milk. Therefore, NFDM formulations were also compared to JK buffer control to focus on impacts of milk protein on flavan-3-ol bioaccessibility while minimizing observations of changes in bioaccessibililty due to presence of milk mineral.

Total flavan-3-ol levels in NFDM DG (15.32±0.38 μmol/digestion reaction) were not significantly different ($p>0.05$) than JK buffer control DG (16.86±0.66 μmol/digestion reaction), but were significantly higher than dd water control (8.64±0.50 μmol/digestion reaction) (Table 9). Interestingly, the overall trend for impact on individual flavan-3-ol bioaccessibility by formulation of green tea extract beverages with NFDM was different depending on whether milk mineral buffer was present (Figure 7, Table 10). Flavan-3-ol bioaccessibility generally increased with addition of NFDM compared to dd water control, but decreased compared to buffer control. Levels of EGC and EGCG in NFDM based beverage DG were 9.72±0.25 and 2.62±0.27 μmol/digestion reaction (Figure 6, Table 10) translating to significantly improved ($p<0.05$) bioaccessibilities of EGC and EGCG from NFDM formulations compared to dd water control from 30.2±0.9 and 7.1±0.2% (59.4±1.8 μmol/g and 16.9±0.5 μmol/g) to 71.9±1.4 and 13.1±1.2% (141.6±2.7 and 31.4±2.4 μmol/g), respectively (Figure 7, Table 10). EC and ECG were present in NFDM DG at 2.5±0.1 and 0.5±0.1 μmol/digestion reaction (Figure 6). Bioaccessibilities of EC and ECG from NFDM formulations were 68.7±2.9% (25.7±5.7 μmol/g) and 10.9±1.1% (4.4±2.1 μmol/g), respectively and were not significantly different ($p>0.05$) compared to dd water based tea beverage control (Figure 7, Table 10).

Compared to buffer control, bioaccessibility of EGC was significantly ($p<0.05$) higher, increasing from 47.5±3.0% to 71.9±1.4% (98.3±9.2 μmol/g to 141.6 μmol/g) by addition of NFDM (Figure 7, Table 10). However relative bioaccessibility of EC, EGCG, and ECG were significantly ($p<0.05$) decreased to 68.7±2.9, 13.1±1.2, and 10.9±1.1% from 82.6±1.4, 16.9±0.5 and 16.1±0.4%, respectively, compared to JK buffer controls by addition of NFDM (Figure 7, Table 9). In terms of absolute bioaccessibility, EC, EGCG, and ECG decreased to 25.7±5.7, 31.4±2.4, and 4.4±2.1 μmol/g from 30.9±0.5, 40.5±1.2, and 6.51±0.2 μmol/g, respectively, compared to JK buffer controls by addition of NFDM.

Although there was no significant difference ($p>0.05$) in flavan-3-ol bioaccessibility from green tea beverages formulated with 10, 20, and 40% of test material ($p<0.05$) (See Appendix Figure A.1), it appears that relative and absolute bioaccessibility of EGC from green tea beverages formulated with 40% NFDM formulations (71.9±1.4% and 141.6±2.7 μg/g) are higher than formulations containing 10% of the test material (57.5±1.4% and 113.2±2.7 μg/g).

3.4 Green Tea Flavan-3-ol Bioaccessbility in Sodium Caseinate (S-CSN), Milk Mineral, and Water Formulations

Effects of S-CSN formulated in dd water and S-CSN formulated in JK buffer on tea flavan-3-ols were compared to dd water and JK buffer controls, respectively. In addition, differences between S-CSN treatments were investigated to better define the role of protein in presence and absence of milk minerals. S-CSN was chosen as a model to investigate impact of milk serum on interactions between milk protein and flavan-3-ols because casein is the milk protein most reported to interact with flavan-3-ols based on

examination of literature (Arts et al. 2002; Lorenz et al. 2007; Shpigelman et al. 2012; Hasni et al. 2011; Kanakis et al. 2011; von Staszewski et al. 2012; Bohin et al. 2012). Additionally, S-CSN in JK (S-CSN/JK) formulation was compared to NFDM to determine extent to which S-CSN may be responsible for observed NFDM effects. Interestingly, flavan-3-ol bioaccessibility from beverages formulated with S-CSN was significantly ($p<0.05$) lower than beverages formulated with NFDM. In regards to impact of milk mineral buffer on S-CSN-flavan-3-ol interactions, there appears to be no overall effect.

Total flavan-3-ols level in S-CSN/JK DG (5.91±0.45 μmol/digestion reaction) was significantly lower ($p<0.05$) than JK control (16.86±0.66 μmol/digestion reaction) (Table 9). S-CSN/JK had significantly ($p<0.05$) decreased DG levels of each individual flavan-3-ol compared to buffer control (Figure 6, Table 10). Specifically, levels of EGC, EC, EGCG, and ECG in S-CSN/JK DG were 1.40±0.42, 2.74±0.01, 1.39±0.16, 0.16±0.02 μmol/digestion reaction. Accordingly, relative bioaccessibilities of EGC, EC, EGCG, and ECG from beverages formulated with S-CSN/JK were significantly lower ($p<0.05$) compared to JK control decreasing from 49.9±4.7, 82.6±1.4, 16.9±0.5, and 16.1±0.4% to 9.1±2.4, 58.1±0.6, 4.7±0.6, and 7.1±0.6% (Figure 7, Table 10). In absolute terms, S-CSN/JK significantly ($p<0.05$) decreased bioaccessibility of all flavan-3-ols compared to JK control from 98.3±9.2, 30.9±0.5, 40.5±1.2, and 6.5±0.2 μmol/g to 17.8±4.7, 21.7±1.2, 11.3±1.2, and 2.9±1.2 μmol/g respectively for EGC, EC, EGCG and ECG.

Total flavan-3-ols level in aqueous S-CSN (S-CSN/water) DG (5.42±0.26 μmol/digestion reaction) was not significantly different ($p>0.05$) than dd water control (8.64±0.50 μmol/digestion reaction) (Table 9). However, beverages formulated with S-

CSN/water had significantly lower and higher ($p<0.05$) DG levels of EGC and EC, respectively, compared to dd water control beverages with 0.86±0.24 and 3.00±0.07 relativeto 4.08±0.49 and 2.55±0.03 μmol/digestion reaction EGC and EC, respectively (Figure 6, Table 10). There was no significant difference ($p>0.05$) in DG levels of EGCG and ECG from S-CSN/water formulations (1.14±0.05 and 0.42±0.00 μmol/digestion reaction, respectively) compared to dd water control beverages (1.63±0.08 and 0.39±0.01 μmol/digestion reaction, respectively). In terms of bioaccessibility, S-CSN/water significantly decreased ($p<0.05$) bioaccessibility of only EGC compared to dd water control from 30.2±0.9 to 6.8±1.0% (59.4±1.8 to 13.5±2.0 μmol/g), but did not significantly ($p>0.05$) affect bioaccessibility of remaining flavan-3-ols compared to control (Figure 7, Table 10).

Individual flavan-3-ol levels in S-CSN/JK DG were not significantly different ($p>0.05$) from levels in S-CSN/water DG (Figure 6, Table 10). Similarly, relative and absolute bioaccessibilities of each flavan-3-ol from S-CSN/JK compared to S-CSN/water formulations were not significantly different ($p<0.05$) (Figure 7, Table 10). Compared to NFDM, both S-CSN/JK and S-CSN/water formulations decreased relative bioaccessibility of EGC and EGCG following *in vitro* digestion to 9.1±2.4 and 4.7±0.6% (17.8±4.7 and 11.3±1.2 μmol/g) from beverages formulated with S-CSN/JK, and to 6.8±1.0 and 4.9±0.1% (13.5±2.0 and 11.7±0.1 μmol/g) from beverages formulated with S-CSN/water. In addition, both EC and ECG bioaccessibility were significantly decreased ($p<0.05$) by formulation withS-CSN/JK buffer compared to formulation with NFDM from 68.7±2.9 and 10.9±1.1% (25.7±5.7 and 4.4±2.1 μmol/g) to 58.1±0.6 and 7.1±0.6% (21.7±1.2 and 2.9±1.2 μmol/g) respectively (Figure 7, Table 10).

Interestingly, although there was no significant dose dependent effect ($p>0.05$) on bioaccessibility of each flavan-3-ol for any treatment tested, it appears that relative and absolute bioaccessibility of EGC from green tea beverages formulated to 40% with S-CSN (9.1±2.4% and 17.8±4.7 μg/g for S-CSN/JK buffer, 6.8±1.0% and 13.5±2.0 μg/g for S-CSN/water) are lower than formulations formulated to 10% of the test material (22.6±2.7% and 44.4±5.2 μg/g for S-CSN/JK buffer, 26.4±3.1% and 52.1±6.1 μg/g for S-CSN/water) (Figure A.2).

3.5 Green Tea Flavan-3-ol Bioaccessibility in Beverages Formulated with Individual Whey Proteins and Milk Minerals

The impact of individual whey proteins on flavan-3-ol bioaccessibility was investigated. Both α-LA and β-LG were formulated in JK buffer at levels that represent those found in fat-free milk to better compare effects of each protein relative to JK buffer control, NFDM and S-CSN/JK formulations.

Total flavan-3-ols level in α-LA in buffer DG (13.90±0.74 μmol/digestion reaction), β-LG in buffer DG (14.48±0.28 μmol/digestion reaction) and buffer control DG (16.86±0.66 μmol/digestion reaction) were not significantly different ($p>0.05$) than dd water control (8.64±0.50 μmol/digestion reaction) (Table 9). With regards to individual flavan-3-ols, absolute levels of EGC in DG of beverages formulated with α-LA and β-LG (7.12±0.47 and 8.44±0.25 μmol/digestion reaction, respectively) were not significantly different ($p>0.05$) compared to buffer without protein added (7.90±0.64 μmol/digestion reaction) (Figure 8, Table 10). In terms of bioaccessibility, whey proteins had no significant affect ($p>0.05$) on EGC bioaccessibility compared to buffer control (Figure 9,

Table 10). Relative bioaccessibilities of EGC from α-LA and β-LG were 45.5±2.8 and 54.7±0.3%, respectively, and absolute bioaccessibilities were 89.6±5.6 and 107.6±0.6 μmol/g, respectively (Figure 9, Table 10).

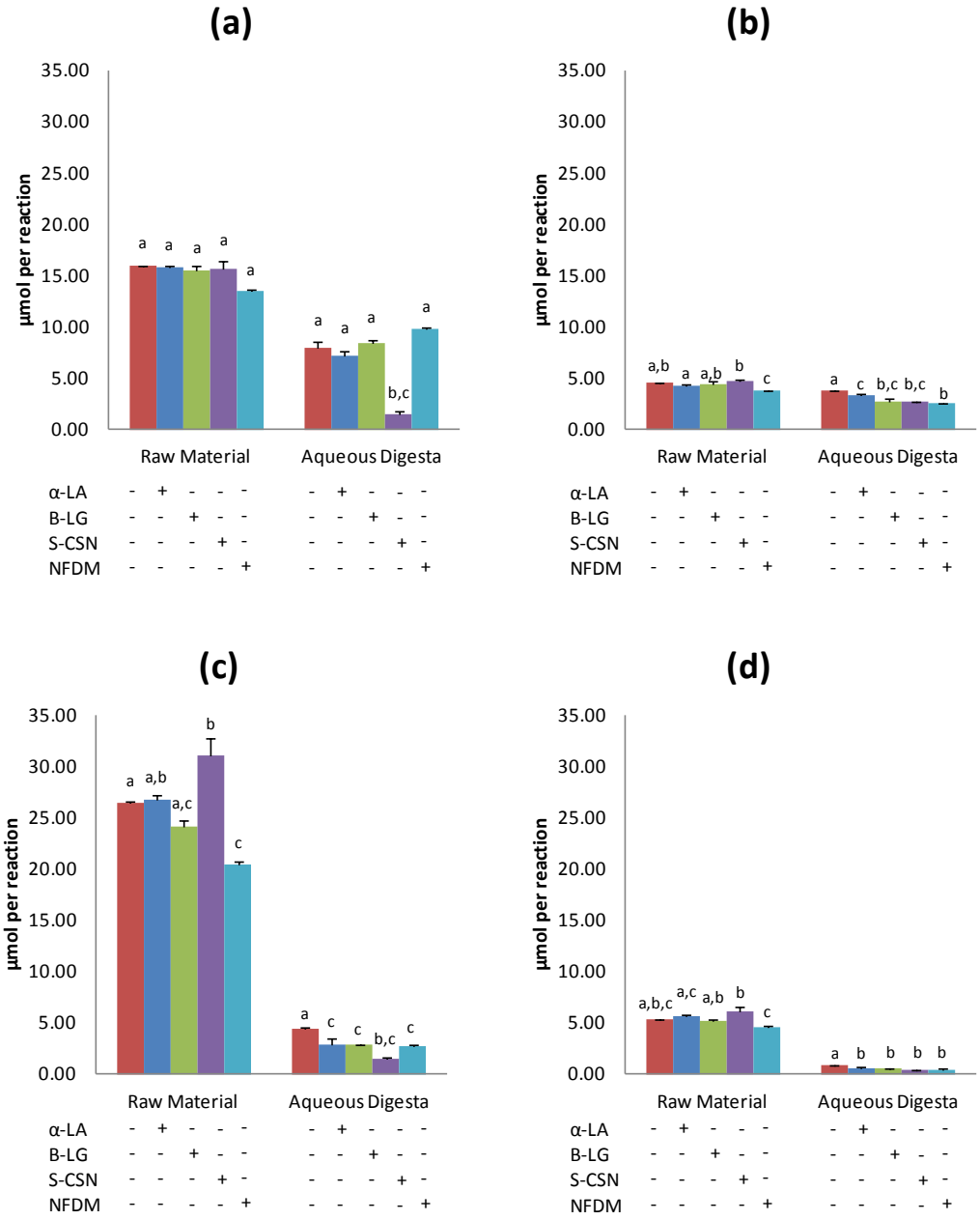


Figure 8: Levels of (a) EGC, (b) EC, (c) EGCG, and (d) ECG in starting material and aqueous digesta of green tea beverages formulated with whey proteins or sodium caseinate in buffer and buffer control. Presence of different letters within raw material and aqueous digesta data indicates significantly different flavan-3-ol content between formulations.

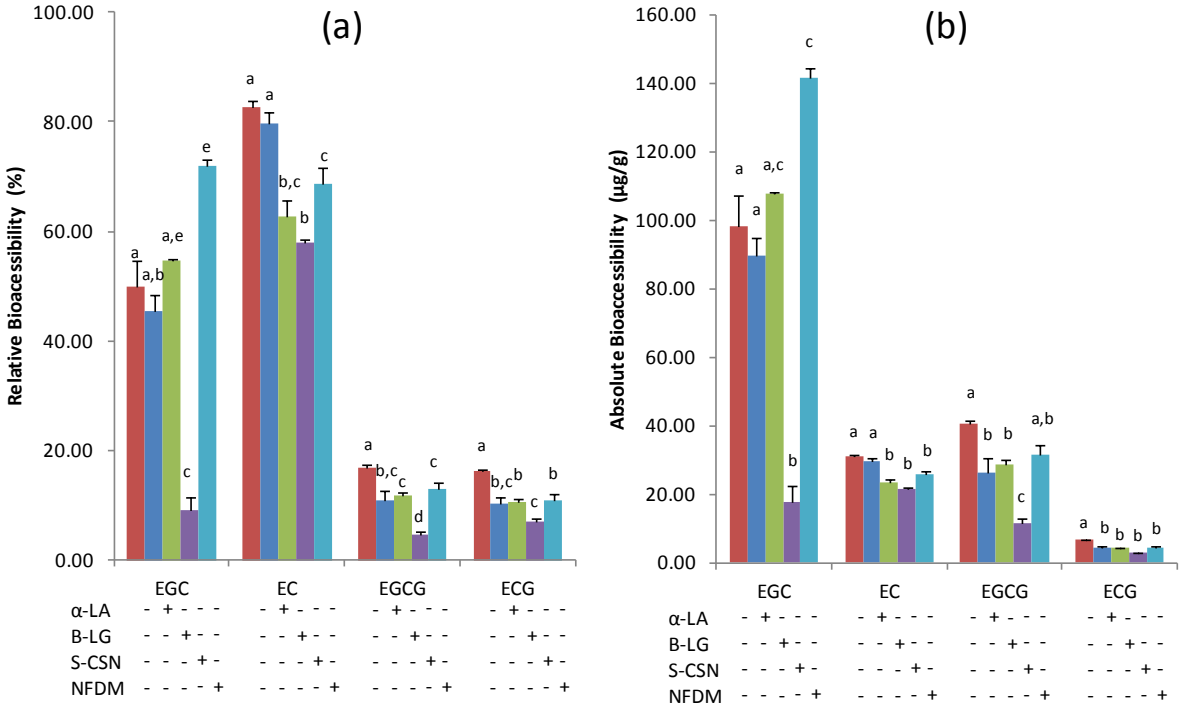


Figure 7: Relative bioaccessibility (%) of flavan-3-ols from green tea beverages formulated with whey proteins or sodium caseinate in buffer and buffer controls. Presence of different letters indicates significantly different flavan-3-ol recovery between formulations.

Beverages formulated with individual whey proteins had significantly decreased DG levels of EC, EGCG and ECG compared to JK control: their respective levels in α-LA DG were 3.34±0.15, 2.89±0.54 and 0.55±0.8 µmol/digestion reaction; and in β-LG DG were 2.73±0.08, 2.81±0.09, and 0.50±0.02 µmol/digestion reaction (Figure 8, Table 10). Bioaccessibilities of EGCG and ECG were significantly ($p<0.05$) decreased by α-LA compared to buffer control to 10.9±1.8 and 10.4±1.1% (26.2±3.5 and 4.2±2.2 µmol/g), respectively (Figure 9, Table 10). However, bioaccessibility of EC from α-LA formulation (79.7±2.0%, 29.8±3.9 µmol/g) was not significantly different ($p>0.05$) compared to buffer control. β-LG significantly decreased ($p<0.05$) bioaccessibilities of

EC, EGCG, and ECG compared to buffer control to 62.8±2.9, 11.9±0.6, and 10.7±0.6%, respectively (23.5±5.6, 28.6±1.1, and 4.3±1.2 μmol/g, respectively).

Flavan-3-ol relative bioaccessibility from whey protein formulated beverages were compared to NFDM formulations to determine extent to which they may be responsible for effects from a more complex NFDM system (Figure 9, Table 10). Compared to NFDM, α-LA significantly ($p<0.05$) decreased relative bioaccessibility of EGC to 45.5±2.8% (89.6±5.6 μmol/g) and significantly increased ($p<0.05$) bioaccessibility EC to 79.7±2.0% (29.8±3.9 μmol/g), but did not significantly ($p>0.05$) affect bioaccessibility of EGCG or ECG. No significant ($p<0.05$) differences in bioaccessibility of individual flavan-3-ols were found between β-LG and NFDM formulations.

Bioaccessibility of flavan-3-ols from whey protein in JK buffer formulations was also compared to bioaccessibility of those from S-CSN in buffer formulations (Figure 9, Table 10). Whey protein formulations had significantly increased ($p<0.05$) bioaccessibilities of gallated flavan-3-ols compared to S-CSN in buffer formulations. In addition, α-LA and β-LG formulations had significantly increased EC and ECG bioaccesibility compared to S-CSN in buffer formulations.

3.6 Protein Digestibility through Simulated GI Model

SDS-PAGE analysis was utilized to characterize extent of protein digestion through the *in vitro* digestion model utilized for the bioaccessibility study (Figure 10). According to preliminary results, the most prominent bands for each raw material used in this investigation correspond to protein with the following molecular weights: <14.4 kDa for α-LA; 14.4 kDa for β-LG; and 25 and 30 kDa for S-CSN. Prominent bands for

background enzymes correspond to protein with molecular weights ranging from 25 to 70 kDa for pancreatin, and 66 kDa for pepsin (See Appendix Figure B.1). For all protein digestas, it appears that after 1 hour of gastric digestion bands resulting from separation of undigested digested protein are present, but at lower concentrations. Furthermore, it appears that protein had been digested to form lower molecular weight peptides. Specifically, it appears proteins present in S-CSN G0 (undigested) range from 14-35 kDa, with most of them between two large bands: one around 25 and one around 30 kDa. All proteins above 14 kDa disappeared after one hour of digestion (S-CSN G1), and thick, faint protein bands appeared below 14 kDa. β-LG G0 had one prominent protein band just above 14 kDa, which corresponds to β-LG monomer (molecular weight 18.4 kDa) in agreement with other studies (J. Mouécoucou et al. 2004; Mandalari et al. 2009; Otte et al. 1997). This band is present in β-LG G1, but is less prominent. These results indicate some level of protein digestion during gastric phase consistent with exposure to pepsin. Changes in protein profile of α-LA, however, are harder to distinguish due to poor resolution of low molecular weight proteins by the type of gel used in this study.

For all milk proteins, bands present following gastric digestion disappeared after just 15 minutes of small intestinal digestion. This indicates that proteins and large polypeptides were rapidly digested to molecular weights less than 10 kDa from exposure to pancreatin during small intestinal digestion (Figure 10).

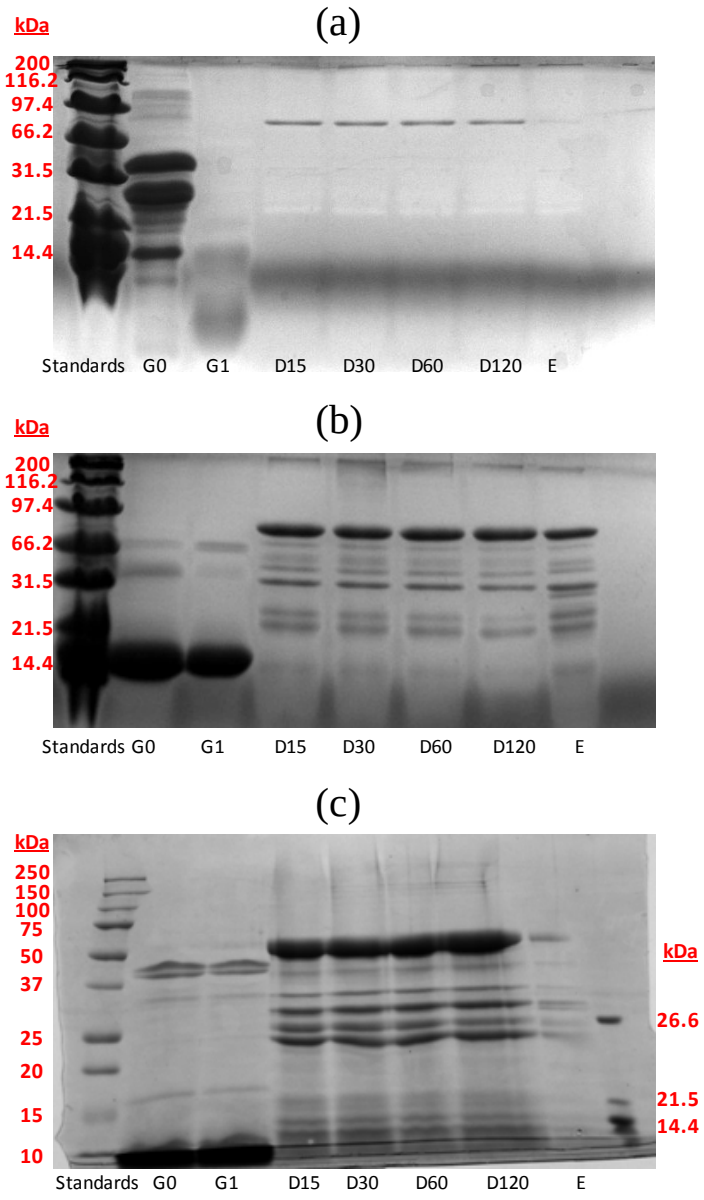


Figure 10: Protein profile of (a) S-CSN, (b) β-LG, and (c) α-LA digests. The following are the lane designations for each gel: Lane 1 (and lane 9 in Figure 1.C): molecular weight markers; lanes 2 and 3: peptic digests (0 and 1 hour incubation, respectively); lanes 4-7: pancreatic digests (15, 30, 60, and 120 minutes incubation, respectively); lane 8: background enzymes (pepsin, pancreatin).

3.7 Characterization of Milk Protein-EGCG Binding by Fluorescence Spectroscopy

Conditions of digestive environment and protein digestion may impact interactions between flavan-3-ols and protein/peptides. As sodium caseinate is the most abundant protein in milk, it was chosen as the model protein to investigate: (1) S-CSN-EGCG binding interactions facilitated by digestive pH (gastric pH 3.0, duodenal pH 6.5) (2) extent of binding between EGCG and digestive enzymes and (3) combined effect of protein digestion, pH, and presence of enzymes in S-CSN digesta on fluorescence quenching of protein Trp residues by EGCG (Table 11, Figure 12).

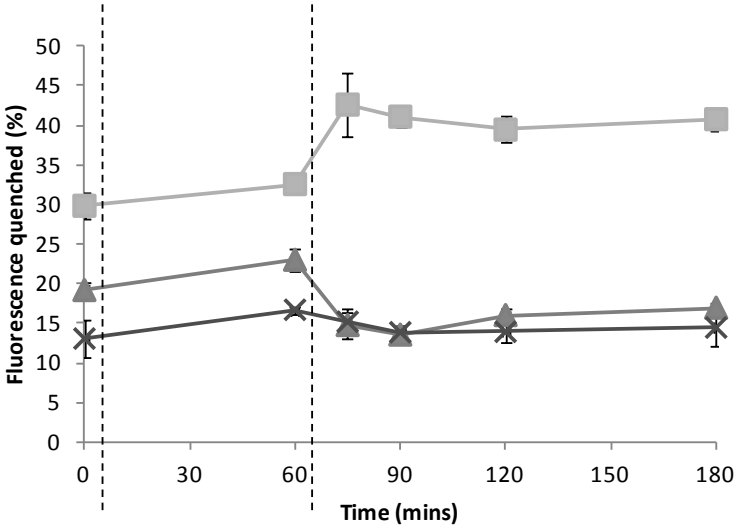


Figure 12: Percent Trp fluorescence quenched of protein solutions by EGCG. S-CSN solutions and EGCG (12μM) or Tris HCl were mixed 1:1 for analysis by fluorescence spectroscopy. Each line represents one of three different procedures used to determine: (▲) extent of binding between EGCG and digestive enzymes, (■) S-CSN-EGCG binding effects due to digestive pH (gastric pH 3.0, duodenal pH 6.5), and (✕) combined effect of protein digestion, pH, and presence of enzymes in S-CSN digesta on fluorescence quenching of protein Trp residues by EGCG. The area of the graph before the first vertical dotted line corresponds with untreated protein, followed by protein incubated at gastric conditions, and protein incubated at duodenal conditions.

Table 11: Percent Trp fluorescence quenched by EGCG of protein solutions. Protein solutions and EGCG (12μM) or Tris HCl were mixed 1:1 for analysis by fluorescence spectroscopy. Each line is one of four different procedures used to determine: (1) extent of binding between EGCG and digestive enzymes (2) S-CSN-EGCG binding effects due to digestive pH (gastric pH 3.0, duodenal pH 6.5) and (3) combined effect of protein digestion, pH, and presence of enzymes in S-CSN digesta on fluorescence quenching of protein Trp residues by EGCG. Different letters between digests within a treatment denotes significant difference ($p<0.05$).

Treatment	Incubation	Fluorescence Quenched (%)
(1) Enzymes, pH 3 and 6.5	G0	$19.2 \pm 1.1^{a,b}$
	G1	23.0 ± 1.4^{a}
	D15	$14.8 \pm 1.6^{b,c}$
	D30	13.6 ± 0.4^{c}
	D60	$16.0 \pm 0.8^{b,c}$
	D120	$17.0 \pm 0.6^{,c}$
(2) S-CSN, no enzymes, pH 3 and 6.5	G0	29.8 ± 1.6^{a}
	G1	$32.6 \pm 0.5^{a,c}$
	D15	42.6 ± 3.9^{b}
	D30	41.1 ± 1.4^{b}
	D60	$39.5 \pm 1.7^{b,c}$
	D120	$40.8 \pm 1.5^{b,c}$
(3) S-CSN, enzymes, pH 3 and 6.5	G0	13.1 ± 2.4^{a}
	G1	16.7 ± 0.5^{a}
	D15	15.2 ± 1.6^{a}
	D30	13.9 ± 0.9^{a}
	D60	13.9 ± 1.4^{a}
	D120	14.5 ± 2.2^{a}

$\%F_q$ of S-CSN incubated at gastric pH and 37°C for 0 or 1 hr (GpH0, GpH1) was 29.8±1.6 and 32.6±0.5% (Table 11, Figure 12). $\%F_q$ of S-CSN incubated at duodenal pH and 37°C for 15 min to 2 hr (DpH15, DpH30, DpH60, DpH120) was higher in comparison ranging from 39.5±1.7 to 42.6±3.9%. S-CSN GpH0 had significantly lower

($p<0.05$) $\%F_q$ compared to all S-CSN samples incubated at duodenal pH: $\%F_q$ was 29.8±1.6% for GpH0 compared to 42.6±3.9, 41.1±1.4, 39.5±1.7, and 40.8±1.5% for S-CSN DpH15, DpH30, DpH60, and DpH120. However, S-CSN GpH1 $\%F_q$ (32.6±0.5) was significantly lower ($p<0.05$) compared DpH15 and DpH30, but not DpH60 and DpH120. No significant differences ($p<0.05$) were found within gastric or duodenal samples.

$\%F_q$ of enzymes incubated at gastric pH for 0 or 1 hr at 37°C (EG0, EG1) was 19.2±1.1 and 23.0±1.4% (Table 11, Figure 12). $\%F_q$ of enzymes incubated at duodenal pH for 15 to 120 min at 37°C (ED15, ED30, ED60, ED120) was lower, ranging from 13.6±0.4 to 17.0±0.6%. EG1 $\%F_q$ was significantly higher ($p<0.05$) compared to all enzyme samples incubated at duodenal pH: $\%F_q$ was 23.0±1.4% for EG1 compared to 14.8±1.6, 13.6±0.4, 16.0±0.8, and 17.0±0.6% for ED15, ED30, ED60, and ED120. However, EG0 $\%F_q$ (19.2±1.1%) was significantly higher ($p<0.05$) compared to only ED30. No significant ($p<0.05$) differences were found within gastric or duodenal samples.

$\%F_q$ of each digesta resulting from digestion of S-CSN by pepsin for 0 to 1 hour at 37°C and pH 3.0±0.1 (FG0, FG1) and by pancreatin for 15 to 120 minutes at 37°C and pH 6.5±0.1 (FD15, FD30, FD60, FD120) ranged from 13.1±2.4 to 16.7±0.5% (Table 11, Figure 12). There were no significant differences ($p<0.05$) in $\%F_q$ between samples. FG0, FG1, FD15, FD30, FD60, and FD120 $\%F_q$ were 13.1±2.4, 16.7±0.5, 15.2±1.6, 13.9±0.9, 13.9±1.4, and 14.5±2.2%.

$\%F_q$ from S-CSN samples incubated without enzyme (GpH0 to DpH120) were significantly higher ($p<0.05$) compared to all enzyme samples incubated without protein (EG0 to ED120) and all S-CSN digesta pH adjusted to gastric or duodenal pH before

fluorometric analysis (FG0 to FD120) (See Appendix Table C.4). However, $\%F_q$ EG0 and EG1 was higher compared to only FG0 and FG1, but the difference was not significant ($p>0.05$).

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CHAPTER 4. DISCUSSION

Prior studies have investigated binding interactions between monomeric flavan-3-ols and proteins derived from foods and from the body (ie. digestive enzymes). With regards to milk proteins, evidence suggests that α- and β-CSN, α-LA, and β-LG all have capacity to interact with EGCG, as well as other flavan-3-ols, binding non-specifically with various affinities (Almajano et al. 2007; Hasni et al. 2011; Kanakis et al. 2011; von Staszewski et al. 2012; Bohin et al. 2012; Jöbstl et al. 2006; Tantoush et al. 2012; Prigent et al. 2009). Non-specific binding of milk proteins with flavan-3-ols occurs primarily through hydrophobic interactions (driven by London dispersion interactions) and hydrogen bonding interactions and, to a lesser extent, electrostatic interactions. Interactions tend to increase with polyphenol molecular weight, hydrophobicity, galloylation, hydroxyl groups, and decrease with methylation, methoxylation, glycosylation, and hydrogenation of the C2=C3 double bond (Soares et al. 2007; de Freitas et al. 2001; Baxter et al. 1997; Xiao et al. 2011; Xu & Chen 2011; Papadopoulou et al. 2005). Protein characteristics that promote interactions with polyphenols include presence of proline, histidine, arginine, phenylalanine and hydrophobic pockets and residues (Yan et al. 1995; Naurato et al. 1999; Charlton et al 2002; Baxter et al. 1997; Charlton et al. 2001; Soares et al. 2007; Papadopoulou et al. 2005). Types and strengths of interactions between specific flavan-3-ols and milk proteins have been outlined in

Table 2 (See Section 1.6.2). Implications of such interactions in food extend to the GI after consumption as non-specific binding may alter availability of flavan-3-ols for reactions and absorption. While limiting accessibility for reactions, binding may serve to stabilize polyphenol with respect to potential oxidative degradation, and strong binding may serve to make these phenolics less available for absorption (bioaccessibility). Therefore understanding extent and implications of such interactions is needed in order to optimize stability but also availability of polyphenols for delivery of health benefits.

The ability of protein to influence flavan-3-ol bioavailability remains controversial. While minimal impact on bioavailability of flavan-3-ols has been observed in clinical studies with black tea based systems (van het Hof et al. 1998; Kyle et al. 2007), in cocoa products, presence of milk protein appears to have a negative effect on monomeric flavan-3-ol bioavailability (Serafini et al. 2003; Neilson et al. 2009). The influence of protein on tea flavan-3-ol bioactivity however, has been observed. Milk had no effect on plasma antioxidant activity following consumption of green or black tea by humans *in vivo* (Leenan et al. 2000), however milk decreased vasorelaxation effects of black tea in humans (Lorenz et al. 2007). While many studies attribute changes in bioactivity to reduced bioavailability, this effect is unclear. Further, the impact of minerals derived from milk on flavan-3-ol stability and bioavailability has not been truly explored. Considering the important interplay between protein-minerals, minerals-flavan-3-ols and flavan-3-ols-protein, additional insight is needed. The purpose of this study was therefore to determine effects of key milk proteins individually and in NFDM matrix as well as milk minerals, in the form of JK buffer, on digestive stability and ultimate

bioaccessibility of green tea flavan-3-ols. As a secondary objective extent to which non-specific binding of flavan-3-ols to milk protein during digestion was assessed by fluorometry.

Relative (%) and absolute (μg/g) bioaccessibility was calculated for green tea beverages formulated with three different concentrations (10, 20, 40%) of each test material. There was no significant ($p>0.05$) concentration effect for any treatment tested (Figures A.1-A.4). Therefore, it is important to note that this discussion will be based primarily on bioaccessibility of flavan-3-ols from green tea powdered extract formulated into model beverages with 40% test material (protein or buffers).

4.1 Impact of Milk Minerals on Flavan-3-ol Bioaccessibility

Green tea powder was formulated with either JK buffer or dd water into model beverage systems and subjected to *in vitro* digestion to determine the impact of milk minerals on digestive stability of flavan-3-ols. Auto-oxidative degradation of flavan-3-ols during gastrointestinal digestion has been previously reported (Yoshino et al. 1999; Zhu et al. 2002; Green et al. 2007; Neilson et al. 2007; Ferruzzi et al. 2010). These reactions are facilitated by the elevated pH of the small intestine and rely on reactivity of the flavan-3-ol B-ring making EGC and EGCG more suceptable to oxidative loss by virtue of their galloyl structure relative to the catechol structure of EC and ECG (Neilson et al. 2007). Degradation of flavan-3-ols in model beverages formulated with dd water was consistent with those previously reported for tea (Green et al., 2007; Neilson et al., 2007; Record & Lane, 2001). Interestingly, bioaccessibilities of all flavan-3-ols were significantly improved by addition of milk mineral rich JK buffer to beverage

formulation ($p<0.05$) relative to dd water (Figure 7, Table 10). This effect waslikely due to presence of divalent metal ions including calcium (Ca) and magnesium (Mg) at levels of 35.9 mg/100mL (9 mM) and 7.8 mg/100mL (3.3 mM), respectively, in the JK buffer system utilized in these studies. These minerals have been previously shown to interact with polyphenols in various foods altering both mineral stability and bioaccessibility (Vitali et al. 2008; Al-Numair 2009).

In terms of potential polyphenol interactions, several studies have reported interactions between catechols, flavonoids and select metals (Namasivayam & Sumithra 2004; Turkel et al. 2004). Polyphenols are well known as metal chelators (Hagerman et al. 2003; Sugihara et al. 2001; Hider et al. 2001; Kumamoto et al. 2001). Results from IR analysis (Yamada et al. 2007) suggest that calcium chelation occurs by either two hydroxyl groups within catechols, commonly present on the B-ring of flavan-3-ols, or by calcium bridging formations between multiple catechols. Flavonoids complexed in such a manner with metal were reported to be less susceptible to oxidation compared to uncomplexed flavanoids (Kostyuk et al. 2004). As previously stated, auto-oxidation is believed to be the primary degradative pathway for flavan-3-ols in the gut lumen due, in part, to the elevated pH of the intestinal compartment (Green et al. 2007; Nielson et al. 2007). In the case of the present study it is highly likely that milk minerals provide a measure of stabilization through their ability to reduce oxidative reactivity of individual tea flavan-3-ols, including highly susceptible EGC and EGCG. These control experiments were thus critical in our ability to distinguish effects in more complicated milk protein systems that also contain significant amounts of minerals that may compete for interactions with flavan-3-ols and alter their stability and subsequent bioaccessibility.

4.2 Impact of NFDM on Flavan-3-ol Bioaccessibility

In order to determine the impact of milk proteins on flavan-3-ol bioaccessibility we first formulated green tea beverages with reconstituted NFDM (10-40%) generating products similar to 10-40% single strength milk addition to tea. These model beverages were compared to green tea beverages formulated with dd water to determine effects of the entire milk matrix on flavan-3-ol digestive stability and to evaluate the relationship with that reported by previous investigators (Green et al. 2007; van der Burg-Koorevaar et al. 2011; Xie et al. 2012). Tables 4 and 5 outline beverage type and polyphenol and milk protein content used for aforementioned *in vivo* and *in vitro* protein-polyphenol interaction studies. Beverages formulated with NFDM were found to have significantly higher ($p<0.05$) relative and absolute bioaccessibility of EGC and EGCG compared to those formulated with dd water, however, no significant impacton bioaccessibility of EC and ECG was observed (Figure 7, Table 10). Green et al. (2007) previously reported that formulation of green tea beverages (49 mg/mL flavan-3-ols) with up to 50% milk improved bioaccessibility of EGC and EC, with no impact to bioaccessibility of EGCG, and decreasing bioaccessibility of ECG. However, addition of milk at 10 and 20% increased bioaccessibility of EGC and was reported to have no effect on bioaccessibility of remaining flavan-3-ols. In another study, formulation of milk to as low as 10% in *boiled* green tea (75 mg/mL flavan-3-ols) decreased bioaccessibility of gallated flavan-3-ols, and formulation of milk up to 25% had no effect on bioaccessibility of EGC and EC. However, formulation of milk to 10% in *ambient* temperature green tea decreased bioaccessibility of EC, and an increase of milk in formulation to 25% decreased bioaccessibility of gallated flavan-3-ols. While our current findings are similar to those

previously described, beverage preparation and temperature may be responsible for subtle differences in reported interactions and impacts of these protein-flavan-3-ol interactions (Xie et al. 2012). Further, inconsistencies in results between studies discussed here may be due to differences in experimental design including: type of digestion (three-stage vs. two-phase); level of digestive enzymes used;environmental conditions (pH of duodenal phase); and formulation (amount and type of tea or milk).

Based on the general chemistry of proteins and flavan-3-ols present in GT-NFDM beverages, there are a few possible explanations for results obtained in our study. Gallated flavan-3-ol bioaccessibility was increased by addition of NFDM compared to controls formulated with dd water, however no effect was found with regards to non-galloylated flavan-3-ols. This may be due to their greater extent of phenolic and hydroxyl groups available for hydrophobic stacking and hydrogen bonding, respectively, with intact protein and/or peptides by galloylated flavan-3-ols. Enhancement in bioaccessibility of select tea flavan-3-ols by addition of NFDM suggest that proteins present during digestion may be binding flavan-3-ols in the product and that these interactions likely continue through initial portions of the digestion process. However, following extensive digestion of protein, flavan-3-ols are released into the gut lumen. Therefore interaction between specific milk proteins and green tea flavan-3-ols may provide some level of protection for individual flavan-3-ols from oxidative degradation in the upper small intestinal phase and increases availability for intact polyphenols to be subsequently absorbed in the upper small intestinal epithelia (bioaccessibility). Increased bioaccessibility may also translate to increased bioavailability as evidence exists to support the notion that intestinal uptake may be altered by presence of protein. Addition

of milk to green tea (69.3 mg/mL flavan-3-ols) to 25% in the formulation increased flavan-3-ol transport across Caco-2 cell membranes compared to beverages formulated with milk to 10% (Xie et al. 2012), and addition of milk protein (2.45 g) to cocoa beverages containing 10 mg/mL cocoa polyhenols slightly accelerated flavan-3-ol absorption in humans (Keogh et al. 2007).

Green tea beverages formulated with NFDM were also compared to those formulated with milk minerals to gain insight into the impact of milk protein relative to effects derived by interactions with just milk minerals (Figure 7, Table 10). Compared to beverages formulated with milk minerals, NFDM significantly increased ($p<0.05$) bioaccessibility of EGC, and decreased ($p<0.05$) bioaccessibilities of EC, EGCG, and ECG, suggesting that interactions between galloylated flavan-3-ols and milk proteins may be conserved during digestion, decreasing flavan-3-ol bioaccessibililty relative to interactions with milk minerals in test beverages. However, this could also suggest that milk mineral interactions may result in soluble complexes that are more stable during digestion than milk protein interactions to better preserve stability of flavan-3-ols.

However, these results remain somewhat inconclusive as interactions between milk minerals and protein (as with NFDM) may alter availability for both minerals and proteins that would result in fewer milk mineral-flavan-3-ol interactions or flavan-3-ol-protein interactions and by extension less flavan-3-ol stability through the GI tract. Also, mineral presence is well known to alter protein structure. The impact of milk minerals on casein structure has been extensively reviewed (Swaisgood; Fennema; Payens et al. 1979; Rollema 1992; Shmidt 1980). Minerals, especially calcium, are required to impart stability to the casein micelle. Studies have shown that changes in variations in mineral

content and casein net charge alter casein structure (Ouanezar et al. 2012; Moitzi et al. 2011). In addition, studies show that minerals may modulate hydrophobic interactions between whey proteins (Phillips et al. 1991), reduce their charge (Schmidt et al. 1978), and aid in formation of whey protein gels (Schmidt et al. 1984). Thus, part of NFDM effect is due to mineral presence. It is therefore likely that observations concerning impact of NFDM proteins on flavan-3-ol bioaccessibility from green tea are more accurate when the treatment is compared to matching controls containing milk minerals than to those lacking milk minerals. Although significant differences in bioaccessibility were found between NFDM treatment and buffer control beverages, differences were modest (about 20, 15, 4, and 5% difference for EGC, EC, EGCG, and ECG, respectively) and thus may not have significant physiological consequences (Figure A.1). Therefore, while these data provide evidence of interactions and modulation of flavan-3-ol bioaccessibility, it would generally agree with previous reports of the minimal impact of milk on polyphenol bioavailability from tea beverages (van het Hof et al.1998; Hollman et al. 2001; Kyle et al. 2007; Keough et al. 2007; Roura et al. 2007; Schramm et al. 2003).

4.3 Impact of Milk Minerals and Individual Milk Proteins on Bioaccessibility of Flavan-3-ols from Green Tea Beverages

Differences in flavan-3-ol bioaccessibility from S-CSN/water compared to S-CSN/JK beverages were investigated to better define the role of protein in presence and absence of milk minerals. There appeared to be no overall effect of milk mineral buffer on S-CSN-flavan-3-ol interactions under the conditions assessed. This may be due to

outcompetition of flavan-3-ol-mineral interactions by strong interactions between S-CSN and flavan-3-ols. The impact of S-CSN/water on flavan-3-ol bioaccessibility was greater compared to milk minerals, supporting this notion (Figure 7).

To determine the impact of individual milk proteins, matching digestions were completed with S-CSN, α-LA, and β-LG solubilized in either dd water or buffer at concentrations reflecting those found in milk replacing NFDM in the formulations. Results from these studies suggest that formulation of JK in place of dd water in green tea beverages altered *in vitro* bioaccessibility of flavan-3-ols. Furthermore, JK contains salts found in milk serum, and thus addition of proteins solubilized in buffer to green tea beverages should allow for a more accurate assessment of how green tea flavan-3-ols and proteins may interact in a milk system. Therefore, two key whey protein in milk (α-LA, and β-LG) were solubilized in JK (1.0 and 3.5 mg/mL, respectively), formulated in green tea beverages between 0.1-0.4 and 0.35-1.4 mg/mL, respectively,and digested *in vitro*. Bioaccessibilities of flavan-3-ols from each type of beverage formulated with an individual milk protein (S-CSN, α-LA, or β-LG) in JK, matching controls, and beverages formulated with NFDM were then compared.

Addition of each individual protein into the final formulation affected bioaccessibility of tea flavan-3-ols. S-CSN in JK significantly decreased bioaccessibility of all flavan-3-ols compared to JK control (Figure 9, Table 10). The micellar structure of casein is disrupted when converted to sodium caseinate and sub-micelle like structures can be formed containing α-s1, α-s2, β, and κ-caseins at 4:1:4:1.3, with the bulk of non-polar regions residing in an inner core and hydrophilic regions on the outer layer (Farrell et al. 1990; Ruis 2007; Pitkowski et al. 2008). Binding of micellar casein and individual

casein constituents to flavan-3-ols has been explored. Caseinomacropeptide was reported to form complexes with green tea flavan-3-ols irrespective of pH (von Staszewski 2012). Additionally, results following ultrafiltration of milk proteins with flavan-3-ols suggest β-casein (β-CSN) has high binding affinity and high binding capacity for EGCG compared to β-LG. β-CSN may have a high affinity for EGCG due to presence of aromatic amino acids and histidines in its structure (Bohin et al. 2012). Results from single molecule force microscopy implied that β-CSN wraps around EGCG, becoming more stiff and compact. The deformation of β-CSN around EGCG was entropic, indicating hydrophobic interactions were the primary force of interaction. Additionally, no enthalpy effects were observed, indicating hydrogen bonding is relatively weak in comparison to hydrophobic interactions (Jöbstl et al. 2006). Another study confirmed through circular dichromism and fluorescence spectroscopy that α- and β-CSNs interact with tea flavan-3-ols through hydrophobic interactions between flavan-3-ol phenolic rings and hydrophobic pockets of casein, altering protein structure resulting in protein unfolding. The authors also suggested that intramolecular hydrogen bonds may tighten the casein structure around the flavan-3-ol (Hasni et al. 2011).

Although the ability of various types of caseins to bind flavan-3-ols has been demonstrated, it is not clear how sodium caseinate interacts with flavan-3-ols. Furthermore, sodium caseinate is very susceptible to pepsin hydrolysis (Guo et al. 1995). It was therefore suprising that these interactions both survived and had effects on ultimate bioaccessibility of flavan-3-ols. To better explore this effect, digestion experiments were designed to test interactions between intact EGCG and milk protein hydrolysates and will be discussed in a later section.

Interestingly, flavan-3-ol bioaccessibility was lower in green tea beverages formulated with S-CSN compared to NFDM formulations. As mentioned above, the structure of casein is significantly disrupted during production of sodium caseinate. On the other hand, casein micelles are partly restored after reconstitution of NFDM (Sharma et al. 1996). Therefore, due to differences in structures between sodium caseinate and casein present in reconstituted NFDM, their potential for interactions with flavan-3-ols may differ. The observed differences in flavan-3-ol bioaccessibility suggest that interactions between sodium caseinate and GT flavan-3-ols may be greater than when casein is present in a more natural state as from reconstituted NFDM or from fresh milk. Therefore, formulation of products should begin to consider not just protein source but form of the protein used if delivery of biologically relevant flavan-3-ols is a key aspect of the product.

Interestingly, this notion was previously observed when comparing the impact of milk and sodium caseinate-based nondairy creamer on bioavailability of phenolics (Renouf et al. 2010). Compared to regular instant coffee, maximum plasma concentration (C_{max}) of chlorogenic acid and metabolites were significantly lower and time needed to reach C_{max} (T_{max}) was significantly longer in coffee beverages formulated with nondairy creamer. However, addition of milk to coffee beverages had no effect on phenolic C_{max} or T_{max}, suggesting potential for fewer or weaker protein-phenol interactions. The primary difference between milk and nondairy creamer is the structure of the casein complex present in the system: milk contains colloidal casein, whereas nondairy creamer typically contains sodium and/or calcium caseinate (Gardiner 1977; Buddemeyer 1980). Similar to our study, it appears that sodium caseinate interacts with phenolics to a greater

extent than casein present in natural micelles, ultimately delaying phenolic intestinal absorption and altering parameters of bioavailability. Although the structure of sodium caseinate is not clear, it can be postulated that more hydrophobic residues are exposed on sodium caseinate compared to casein micelles, strengthening its interaction with flavan-3-ols. As a result, release of flavan-3-ols from complexes with sodium caseinate may occur later than from complexes with casein micelles, therefore increasing time to flavan-3-ol uptake by small intestinal epithelial cells.

With regards to whey proteins, α-LA (assessed at 0.1-0.4 mg/mL) significantly decreased ($p<0.05$) both relative and absolute bioaccessibility of both EGCG and ECG compared to buffer control, but had no significant ($p>0.05$) impact on EGC or EC (Figure 9, Table 10). α-LA has a net negative charge at the pH of milk (pH 6.3-6.6) and folds intramolecularly burying most hydrophobic residues (Swaisgood 1996). This structure would conceivably make hydrophobic sites less available for interaction with phenolic ring structures. Therefore with regards to binding interactions, hydrogen binding should predominate between α-LA and gallated flavan-3-ols in an undigested state. However, α-LA is rapidly degraded by pepsin (Fu et al. 2002). Thus, digested α-LA may expose hydrophobic residues that may in turn associate with gallated flavan-3-ols through GI digestion and result in reduced bioaccessibility.

Recent studies have explored specific interactions between α-LA and flavan-3-ols using SDS-PAGE and particle size analysis (von Staszewski et al. 2011; Tantoush et al. 2012). At pH 4.5, (α-LA's isoelectric point) incubation of whey protein concentrate solutions (8% w/v in dd water) with green tea flavan-3-ols at 0.25% (w/v) for 24 hours resulted in formation and precipitation of large α-LA-flavan-3-ol aggregates. On the other

hand, incubation of the same solution at pH 6.0 generated smaller soluble complexes requiring increased levels of polyphenol (1% w/v) for precipitation (von Staszewski et al. 2011). Another study showed that α-LA (1.0 mg/mL) remained in soluble portion of solutions containing green tea flavan-3-ols (0.2 mg gallic acid equivalents per mL) after 5 minutes of incubation in acetate buffer (pH 4.5). However, α-LA was insoluble in presence of oxidized green tea flavan-3-ols, suggesting rapid formation of insoluble complexes between α-LA and oxidized green tea flavan-3-ols (Tantoush et al. 2012). These results suggest that incubation conditions (time, pH) as well as oxidation state of flavan-3-ols affect extent of flavan-3-ol-α-LA interactions. In the present study α-LA was primarily exposed to pHs above and below its isoelectric point likely resulting in soluble complexes between α-LA and flavan-3-ols, specifically favorable for gallated flavan-3-ols (EGC, EGCG and ECG), explaining, in part, the effect of decreasing their bioaccessibility.

Non-gallated flavan-3-ol (EC) relative and absolute bioaccessibility was not significantly impacted by presence of α-LA in comparison to JK buffer control. Previously, Prigent et al. (2009) reported low affinity of α-LA for EC using isothermal titration calorimetry. It appears that gallation is important in binding between α-LA and flavan-3-ols as it has been reported for other proteins including salivary proteins, casein and albumins (de Freitas et al. 2001; Baxter et al. 1997; Xiao et al. 2011; Xu & Chen 2011; Soares et al. 2007). Over 10% of α-LA amino acid residues are aromatic (Swaisgood 1996) making it plausible that hydrophobic stacking between galloyl groups and aromatic amino acids may be central in α-LA-flavan-3-ol interactions as it is for other proteins mention above. Similar to NFDM, although significant differences in

individual flavan-3-ol bioaccessibility were observed between α-LA treatment and buffer control beverages, it should be noted that these differences were less than 10% (Figure A.2). Therefore, it is not expected that presence of α–LA would have significant impact on bioavailability of flavan-3-ols from tea through binding interactions. The extent to which formulation would drive oxidation and thus loss of absolute bioaccessibility should be considered, however.

β-LG (0.35-1.4 mg/mL) decreased relative and absolute bioaccessibility of EC, EGCG, and ECG compared to JK control (Figure 9, Table 10). As with other protein interactions, this would be consistent with formation and preservation of β-LG-flavan-3-ol associations during digestion. A recent study showed that β-LG complexed with green tea flavan-3-ols, irrespective of pH (von Staszewski et al. 2012). Similar to α-LA, β-LG (8% w/v) and polyphenols (0.25% w/v) interact at pH 4.5 forming large aggregates resulting in precipitation of β-LG from whey protein concentrate. Smaller particles form at pH 6.0, precipitating with addition of higher levels polyphenols (1% w/v) (von Staszewski et al. 2011). Molecular modeling indicated EGCG-β-LG interactions occur through H-bonding networks and hydrophobic interactions between flavan-3-ol phenolic rings and hydrophobic pockets in β-LG (Kanakis et al. 2011). Similar modeling methods suggest ECG-β-LG interactions occur through hydrogen bonding and van der Waals interactions (Wu et al. 2011). Furthermore, structural analysis shows that more EGCG binds to β-LG compared to other green tea flavan-3-ols, and ECG and EGC have the lowest and highest binding constants, respectively (Kanakis et al. 2011). These observations suggest that galloylated flavan-3-ols bind more readily to intact β-LG compared to EC and EGC, consistent with our bioaccessibility results. This would, on

the surface, suggest that binding in the product would be maintained through digestion and impact availability for absorption. Although significant, differences in individual flavan-3-ol bioaccessibility found between β-LG treatment and buffer control beverages, were moderate (about 20, 5, 5, and 6% difference for EGC, EC, EGCG, and ECG, respectively) (Figure A.1). Therefore, as with other whey protein constituents, it is not expected that these interactions would have significant impact on bioavailability of flavan-3-ols from tea through binding interactions in the gut, but rather may drive some changes in bioavailability through stabilization or destabilization of tea flavan-3-ols through beverage formulation.

4.4 Protein Digestibility through Simulated GI Model

In order to confirm the extent of protein degradation by gastric and duodenal digestion, we digested milk proteins to various extents and separated contents of resulting digesta using SDS-PAGE analysis. S-CSN is susceptible to pepsin digestion (Guo et al. 1995) and α-LA is susceptible to pepsin and pancreatin digestion (Fu et al. 2002; Tantoush et al. 2012) while β-LG is somewhat resistant to pepsin and trypsin digestion (Guo et al. 1995; Tantoush et al. 2011). However, digestive conditions in the present studies, including pH, combination of enzymes, and time are different than those used in these previously published studies. Therefore, SDS-PAGE analysis was used to assess the extent to which S-CSN, β-LG, and α-LA were digested in the *in vitro* digestion procedure utilized for flavan-3-ol bioaccessibility studies.

After 1 hour of gastric digestion (G1) of S-CSN, bands resulting from separation of undigested protein (G0) are almost fully digested to about half their original size (Figure

10). Less dramatic effects were observed for β-LG and α-LA digests. β-LG G1 maintained similar protein profile compared to G0, however the level of the prominent protein decreased. This partial resistance to pepsin digestion was previously observed by another group (Guo et al. 1995; Tantoush et al. 2012). α-LA is a smaller protein with molecular weight barely above the limit of separation for the specific gels used for analysis. Therefore, there is very low resolution for the gastric phases, resulting in formation of a dark band at the bottom of the gel for undigested α-LA and α-LA G1, making it difficult to see any changes due to gastric digestion. However previous studies suggest that rapid degradation of α-LA occurs in presence of pepsin (Fu et al. 2002; Tantoush et al. 2012). For all protein digests, after 15 minutes of small intestinal digestion (D15), protein bands present in G0 and G1 digesta disappeared, indicating that peptides were broken down to <10 kDa rapidly from exposure to pancreatin during small intestinal digestion (Figure 10). Based on previous studies, extensive pancreatic digestion was expected for α-LA, but not β-LG (Fu et al. 2002; Tantoush et al. 2011, 2012). β-LG may have been resistant to trypsin digestion (Tantoush et al. 2011) due to the very low trypsin:protein ratio (1:100). However, pancreatin contains other proteolytic enzymes and more accurately reflects the complexity of digestion in the human GI tract. Therefore digestion would be expected in our current model. Also, concentrations of proteases used in our studies were very high: 0.8 and 1.2mg/mL pepsin and pancreatin, respectively, in final digesta. This is about 3x the concentration used in previous studies (Garrett et al. 2000).

Milk proteins appear to be partially degraded during gastric digestion and are rapidly degraded to <10k Da during duodenal digestion at conditions used in our studies (Figure

10). This information may help to explain the increased binding of flavan-3-ols to proteins digested gastrically, and rapid decrease in binding of flavan-3-ols to proteins digested duodenally.

The protein profile of digesta resulting from digestion of solely milk proteins may be very different if digested in presence of flavan-3-ols. Studies have shown that flavan-3-ols bind digestive enzymes, decreasing extent of protein degradation (He et al. 2006; Nakai et al. 2005; Unno et al. 2009). For example, green and oolong tea polyphenols decreased activity of digestive enzymes (He et al. 2006; Nakai et al. 2005; Naz et al. 2011). Therefore tea may alter protein digestibility and thus protein/peptide-flavan-3-ol interactions during digestion.

4.5 Characterization of Milk Protein-EGCG Binding through Digestion

Considering the apparent limited impact of milk proteins on digestive stability/release (bioaccessibility) of flavan-3-ols in our model system, the extent to which binding interactions may be destabilized through digestion was explored. Prior studies have investigated binding of intact flavan-3-ols to milk proteins. Evidence shows that α- and β-CSN, α-LA, and β-LG all interact with flavan-3-ols with various binding affinities (Almajano et al. 2007; Hasni et al. 2011; Kanakis et al. 2011; von Staszewski et al. 2012; Bohin et al. 2012; Jöbstl et al. 2006; Tantoush et al. 2012; Prigent et al. 2009). External quencher accessibility has been previously used as method to assess binding interactions. This method tracks changes in fluorescence tryptophan (Trp) in intact proteins. α- and β-casein contain two and one Trp residues, respectively on their surface

(Kumosinski et al. 1993). Fluorescence spectroscopy was therefore utilized to estimate flavan-3-ol binding through changes in protein Trp fluorescence.

S-CSN was chosen as the model to demonstrate changes in polyphenol-protein binding throughout digestion because it is the most abundant protein in milk. EGCG was chosen as the model flavan-3-ol because it is the most abundant polyphenol in green tea and has high propensity for binding due to its abundance of galloyl and hydroxyl groups available for hydrophobic interactions and hydrogen bonding. Fluorescence of protein in presence of EGCG relative to its fluorescence without EGCG, $\%F_q$, was calculated to determine extent of quenching and by extension "binding" between intact protein or peptides in digesta with EGCG. In the current study we investigated effect of gastric and duodenal pH (pH 3.0 and 6.5, respectively) on flavan-3-ol binding to S-CSN and contribution of Trp fluorescence quenched from background digestive enzymes (pepsin and pancreatin). Finally, all conditions were combined (S-CSN digestion, pH, and enzymes) to determine impact of the full system and compare to impact of each variable (Figure 12).

Protein net charge and conformation change with pH thus altering location of amino acid residues on their structure possibly affecting extent of protein-flavan-3-ol binding. According to results, EGCG quenches significantly more ($p<0.05$) intact S-CSN fluorescence at pH 6.5 compared to pH 3. Also quenching was significantly higher ($p<0.05$) with intact enzyme fluorescence at pH 3.0 compared to 6.5 (Figure 12). This may be due to repulsion between protein and EGCG near the protein's isoelectric point or due to unfavorable charged distribution or conformation of protein for London dipole forces with EGCG. In agreement with aforementioned fluorometry results regarding the

effect of protein digestion on milk protein-EGCG binding, it appears that protein hydrolysis and conformation both are major factors contributing to binding interactions with flavan-3-ols.

Another factor that may impact flavan-3-ol milk protein interactions during digestion is presence of digestive enzymes. Studies have shown that flavan-3-ols interact with digestive enzymes (He et al. 2006; Nakai et al. 2005; Tagliazucchi et al. 2005; Naz et al. 2011). Flavan-3-ol-enzyme interactions may decrease available flavan-3-ols for interaction with milk protein. Therefore, we assessed fluorescence quenching of Trp residues from an enzyme cocktail containing pepsin and pancreatin at pH 3.0 and 6.5. Results indicated that EGCG quenches enzyme Trp fluorescence at both pHs evaluated (Figure 12). Thus, Trp fluorescence quenching observed in our system may be attributable to binding of EGCG with both digestive enzymes and test protein. Unlike S-CSN, however, it appears that enzymes interacted with EGCG to a significantly ($p<0.05$) greater extent at pH 3.0 compared to pH 6.5. Therefore, binding in the duodenal phase were most likely a result of residual interactions with peptides generated from digestion of milk proteins.

An additional factor that may also impact flavan-3-ol binding to milk protein is protein digestion. Intact protein is stabilized by hydrogen bonds and hydrophobic and van der Waals interactions, with hydrophobic areas buried in the center of the molecule. During gastric digestion, proteins are partially denatured by low pH and pepsin digestion. Pepsin is an endopeptidase that hydrolyzes proteins into large peptides. It cleaves peptide bonds preferentially at carboxyl groups of tyrosine, phenylalanine, or tryptophan residues, and does not cleave peptide bonds linked to the imino group of proline. Trypsin and

chymotrypsin are serine endopeptidases in pancreatin that are active during small intestinal digestion. Trypsin preferentially cleaves peptide bonds with positively charged residues, such as arginine or lysine, and proline inhibits cleavage by trypsin. Chymotrypsin acts on bonds with large hydrophobic amino acid residues, such as tryptophan, phenylalanine, tyrosine, methionine, or leucine, in addition to trypsin and chymotrypsin. Pancreatin also contains a general protease, which cleaves peptide bonds non-specifically (Moughan & Stevens 2013). As peptide bonds within protein are cleaved, the structure unfolds uncovering buried polypeptide chains in a process termed demasking (Adler-Nissen 1986). Consequently, amount of ionizable groups is increased, and additional hydrophobic pockets may be exposed (Panyam & Kilara 1996; Vorob'ev, 2013) and likely made available for additional interactions with flavan-3-ols. Furthermore, secondary and tertiary structure of the protein molecule is disrupted, and surface topology and molecular flexibility are altered.

Studies have investigated the impact of milk protein hydrolysis by digestive enzymes on protein structure. With regards to casein, tryptic hydrolysis resulted in protein with lower molecular flexibility and higher α-helix and β-sheet (Wang et al. 2013). Furthermore, hydrophobic residues were increased and ionic residues were decreased by both pepsin and pancreatic hydrolysis, resulting in attraction between protein molecules (Su et al. 2008). Potential structural changes to these proteins induced by digestion may therefore alter their ability to interact with flavan-3-ols. Specifically, hydrophobic interactions, hydrogen binding, and van der Waals interactions (specifically London forces) may be altered favoring additional binding. In general, it appears that after one hour of gastric digestion, the extent to which S-CSN is digested enhances these

interactions with flavan-3-ols (Figure 12). However, after duodenal digestion protein/peptide degradation may be so extensive that potential for strong interactions with flavan-3-ols is decreased. Therefore, while interaction during gastric digestion may be strong, interactions decrease as protein digestion continues to generate small peptides and amino acids during duodenal digestion. Subsequent to binding of protein to flavan-3-ol before or during gastric phase, the time the interaction survives without release will determine influence on flavan-3-ol bioaccessiblity: Release early in the duodenal phase decreases protection and potential for flavan-3-ol interaction with digested protein; release toward end of duodenal phase may increase bioaccessibility; and failure to release may decrease bioaccessiblity. Consequently, bioavailability may be altered modestly. Alternatively, interactions may delay uptake from small intestinal epithelium, increasing T_{max} of flavan-3-ols in plasma, an effect observed for plasma kinetics of chlorogenic acid from coffee by addition of milk in the formulation (Reneuf et al. 2012).

In this study, S-CSN was digested enzymatically and gastric and duodenal samples were pH adjusted to pH 3.0 or 6.5, respectively, before fluorometric analysis to determine the impact of complete digesta environment on Trp fluorescence quenching by EGCG. Results suggest that $\%F_q$ of S-CSN and digestive enzymes are not additive: Trp fluorescence quenched by the whole system was significantly lower ($p<0.05$) at every extent of S-CSN digestion compared to S-CSN incubated under the same conditions without enzyme (Figure 12) at 0 and 1 hour of S-CSN gastric digestion compared to enzyme incubated under the same conditions without S-CSN (Table C.4). Therefore, binding between EGCG with S-CSN and digestive enzymes appears to be non-competitive and non-specific. This suggests that EGCG binds aromatic amino acid

residues besides Trp, and may even bind these residues with greater affinity. In addition, there were no significant ($p<0.05$) differences between $\%F_q$ of each phase of S-CSN digestion. Therefore, variations in fluorescence quenching of S-CSN and enzyme Trp residues observed due to pH and extent of S-CSN digestion may mitigate differences in binding of EGCG to protein/peptides throughout digestion. Although binding is occurring between EGCG and proteins in each digesta, it is hard to distinguish to what extent the impact is due to S-CSN or digestive enzymes. By comparing fluorescence data with SDS-PAGE data, it can be hypothesized that during the gastric phase of digestion, S-CSN and its digestion products may bind EGCG appreciatively, however rapid digestion of S-CSN during the duodenal phase may mitigate binding between S-CSN digestion products. Therefore, binding that occurs during duodenal digestion may be primarily attributable to interactions between digestive enzymes and EGCG.

CHAPTER 5. CONCLUSIONS AND FUTURE WORK

5.1 Overall Conclusions

In the present study, bioaccessibility of individual tea flavan-3-ols appeared to be affected by both presence and type of milk protein. Inclusion of S-CSN significantly decreased ($p<0.05$) relative and absolute bioaccessibility of all flavan-3-ols, β-LG significantly decreased ($p<0.05$) relative and absolute bioaccessibility of all flavan-3-ols except EGC, and α-LA significantly decreased ($p<0.05$) relative and absolute bioaccessibility of only gallated flavan-3-ols compared to buffer controls (Figure 9, Table 10). We attribute these digestive losses to protein-flavan-3-ol interactions that balance both availability of flavan-3-ols for oxidative reactions in the gut environment and solubility of flavan-3-ol-protein complexes. That being said, the impact α-LA and β-LG had on flavan-3-ol bioaccessibility was moderate, while S-CSN had a greater impact. This is consistent with the body of literature on binding interactions between individual proteins and flavan-3-ols in model systems (Hasni et al. 2011; Kanakis et al. 2011; Bohin et al. 2012; Arts et al. 2002; Lorenz et al. 2007; Shpigelman et al. 2012).

Perhaps most interesting, it appears that milk minerals may have more of an effect on flavan-3-ol bioaccessibility than milk proteins themselves. Beverages formulated with JK buffer containing milk minerals significantly increased ($p<0.05$) bioaccessiblity of all flavan-3-ols compared to dd water control (Figure 7, Table 10). However, in presence

of protein, milk minerals appear to lose their ability to affect flavan-3-ol bioaccessibility, possibly due to loss of mineral-flavan-3-ol interactions to competition with mineral-protein interactions (Figure 9, Table 10).

Formation of flavan-3-ol-protein complexes appeared to be dependent on structure of both flavan-3-ol and protein. According to structural analysis executed in previous studies (Hasni et al. 2011; Kanakis et al. 2011; Jöbstl et al. 2006; Prigent et al. 2009; Wu et al. 2011) typical interactions involved in complex formation include hydrophobic interactions between phenolic rings of flavan-3-ols with aromatic amino acids and hydrophobic pockets of protein and, to a lesser extent, hydrogen bonding and van der Waals interactions, for example between hydroxyl groups of flavan-3-ols with protein. These interactions may protect flavan-3-ols from oxidative degradation, or render them insoluble in the gut. Protein that appears to have little or no affect on the bioaccessibility of a polyphenol either does not bind well to the particular polyphenol, or their interaction may be broken by proteases during gastric digestion.

SDS-PAGE results from digestion of milk proteins imply that proteins are degraded by pepsin at concentrations used in this study: high molecular weight and low molecular weight proteins are decreased and increased, respectively, after 1 hour of digestion by pepsin (Figure 10).

The impact of pH on intact S-CSN-EGCG and of S-CSN degradation by pepsin and pancreatin digestion on (poly)peptide-flavan-3-ol interactions was investigated to obtain a more detailed picture of these interactions during digestion (Figure 12). According to results, it appears that pH has the greatest impact on S-CSN-EGCG interactions (Figure 12, Table C.4): EGCG binds S-CSN to a significantly greater ($p<0.05$) extent at pH 6.5

compared to pH 3.0. Pepsin and pancreatin also complexed with EGCG, however binding occurred to a significantly greater ($p<0.05$) extent at pH 3.0 compared to pH 6.5. The change in propensity of protein to bind EGCG with pH is most likely due to changes in structure, amino acid distribution, and net charge of protein.

Although changes in protein-EGCG interactions occurred due to change in pH, binding between EGCG and protein resulting from digestion of S-CSN to various extents were not significantly different ($p>0.05$) from each other (Figure 12, Table C.4). This indicates that combination of individual effects mitigates an overall change in protein-EGCG binding between different stages of digestion. Furthermore, although protein/peptide-EGCG binding occurred, it is unclear to what extent these effects are due to EGCG binding to digestive enzymes.

Taken together, *in vitro* digestion, fluorometry, and SDS-PAGE results suggest that milk protein-flavan-3-ol interactions are (1) dependent upon structure of both protein and polyphenol and (2) altered by gastrointestinal digestion. Therefore, interactions between flavan-3-ols and individual milk proteins may alter available flavan-3-ol levels following gastric digestion and delay absorption in the small intestine. Complexes appear to form during the gastric phase, decreasing bioaccessibility of flavan-3-ols and possibly protecting them from degradation by oxidative conditions. Subsequent degradation of protein during duodenal digestion may break these interactions, increasing bioaccessibility of intact flavan-3-ols, thus increasing levels available for absorption in the small intestine. These interactions would be more prominent as the S-CSN content increases. This would explain differences previously observed between bioavailability of phenolics when formulated with S-CSN nondairy creamers compared to milk (Renouf et

al. 2010). In addition to information obtained by *in vitro* digestion regarding protein-polyphenol interactions, it appears that milk minerals interactions merit further investigation as these interaction may influence flavan-3-ol stability during digestion, thereby increasing flavan-3-ol bioaccessibility.

5.2 Future Work

While results from experiments outlined in this thesis suggest that protein-flavan-3-ol complexes may be present in the gut following consumption of green tea with milk decreasing bioaccessibility of flavan-3-ols, the impact of these complexes with regards to bioavailability needs to be elucidated. *In vitro* data obtained from a Caco-2 cell culture model may clarify to what extent uptake, absorption, and metabolism of flavan-3-ols from small intestinal epithelial cells is affected by interactions between digested proteins with flavan-3-ols. In addition, colonic fermentation may be utilized to determine extent to which protein-flavan-3-ol interactions may inhibit catabolism of flavan-3-ols by colonic microflora to phenolic acids that may subsequently be absorbed into the blood stream more efficiently than the flavan-3-ols themselves.

The protein profile of digesta resulting from digestion of solely milk proteins may be very different if digested in presence of flavan-3-ols. Studies have shown that flavan-3-ols bind digestive enzymes, decreasing extent of protein degradation (He et al. 2006; Nakai et al. 2005; Unno et al. 2009). For example, intact and oxidized flavan-3-ols increased and decreased pepsin activity, respectively (Tantoush et al. 2012). Therefore, milk proteins should be digested in presence of flavan-3-ols, and the protein profile of the digesta should be compared to digesta resulting from digestion of milk proteins digested

without flavan-3-ols to determine the extent to which flavan-3-ols alter digestion of proteins. Similarly, milk protein-flavan-3-ol digesta fluorescence can be compared to fluorescence of milk protein digesta mixed with flavan-3-ol digesta to compare binding of digested flavan-3-ols with milk protein digested with and without presence of flavan-3-ols.

Bioactive milk peptides are released from milk proteins by digestive enzymes. Some are resistant to further digestion (Reynolds et al. 1994), while others are broken down further, sometimes to more active peptides (del Mar Contreras et al. 2013; Quirós et al. 2009; Tavares et al. 2011). The impact of green tea flavan-3-ols on digestive release and/or stability of bioactive peptides may be of interest. This could be achieved by either 1) amino acid sequencing to measure presence of established bioactive peptides, or 2) measuring an indicator of disease prevention (angiotensin converting enzyme inhibition, or markers of antithrombotic activity).

LIST OF REFERENCES

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APPENDICES

Appendix A: Affect of Protein Concentration on Bioaccessibility of Green Tea Flavan-3-ols *in vivo*.

Test solutions containing milk proteins were formulated to mimic concentrations naturally occurring in milk (NFDM; α-LA, β-LG and S-CSN in buffer; S-CSN in water) and formulated into green tea beverages at 10, 20, and 40%. There was no significant concentration effect ($p<0.05$) at the three concentrations tested. Therefore we discussed only beverages formulated to 40% with protein solution.

Although there was no significant difference ($p>0.05$) in flavan-3-ol bioaccessibility from green tea beverages formulated with 10, 20, and 40% of test material ($p<0.05$) (Figures A.1, A.2, A.3, A.4), it appears that relative and absolute bioaccessibility of EGC from green tea beverages with formulated with 40% NFDM formulations (71.89±1.36% and 141.57±2.68 μg/g) are higher than formulations containing 10% of the test material (57.50±1.37% and 113.22±2.70 μg/g) (Figures A.1. and A.3) In addition, it appears that relative and absolute bioaccessibility of EGC from green tea beverages formulated to 40% with S-CSN (9.05±2.37% and 17.82±4.68 μg/g for S-CSN in buffer, 6.84±1.01% and 13.48±1.98μg/g for S-CSN in water) are lower than formulations formulated to 10% of the test material (22.55±2.65% and 44.40±5.22 μg/g for S-CSN in buffer, 26.44±3.08% and 52.06±6.06 μg/g for S-CSN in water) (Figures A.2 and A.4).

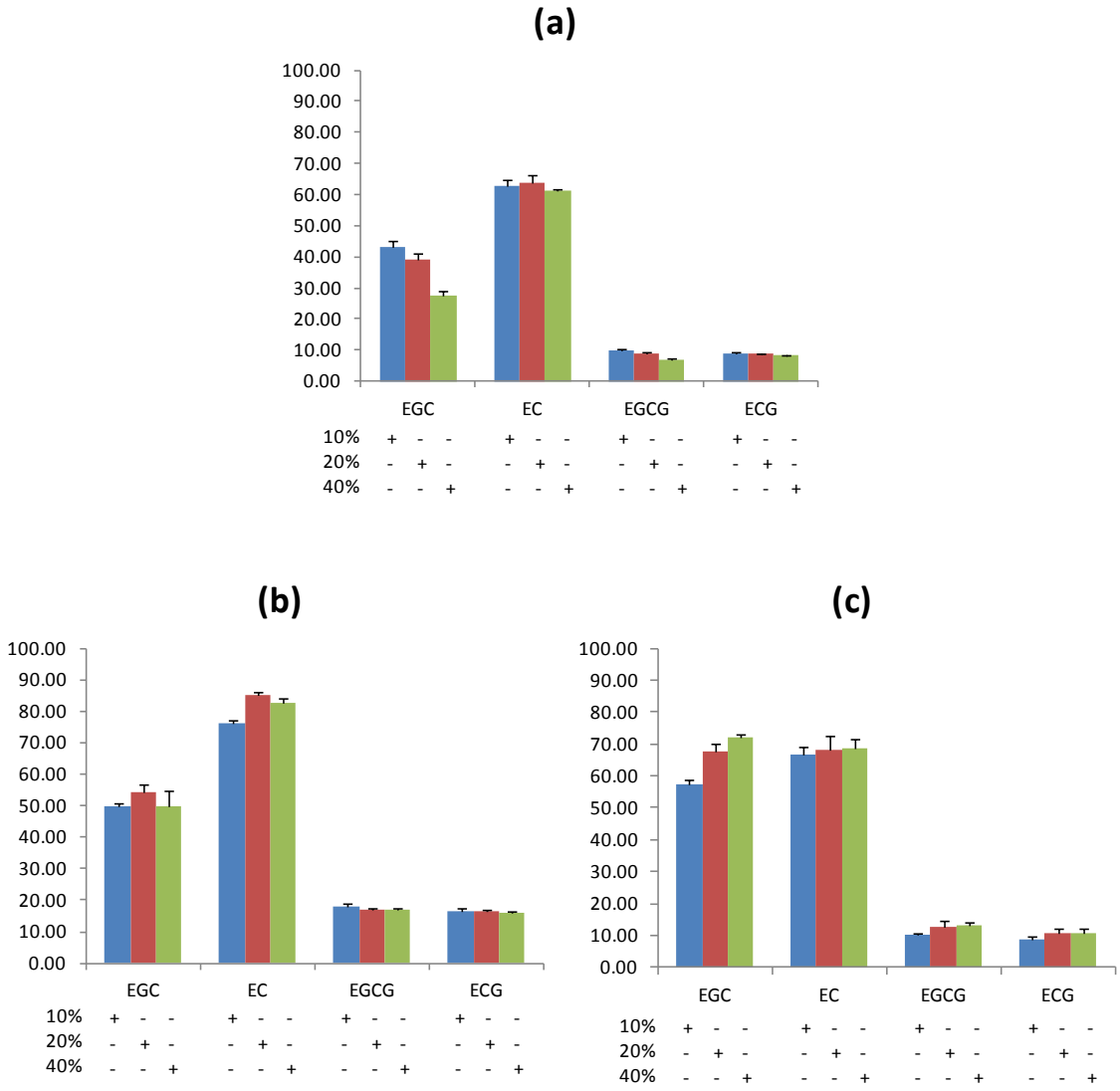

Figure A. 5: Relative bioaccessibility (%) of EGC, EC, EGCG, and ECG from green tea beverages formulated with (a) water, (b) buffer, and (c) nonfat dry milk.

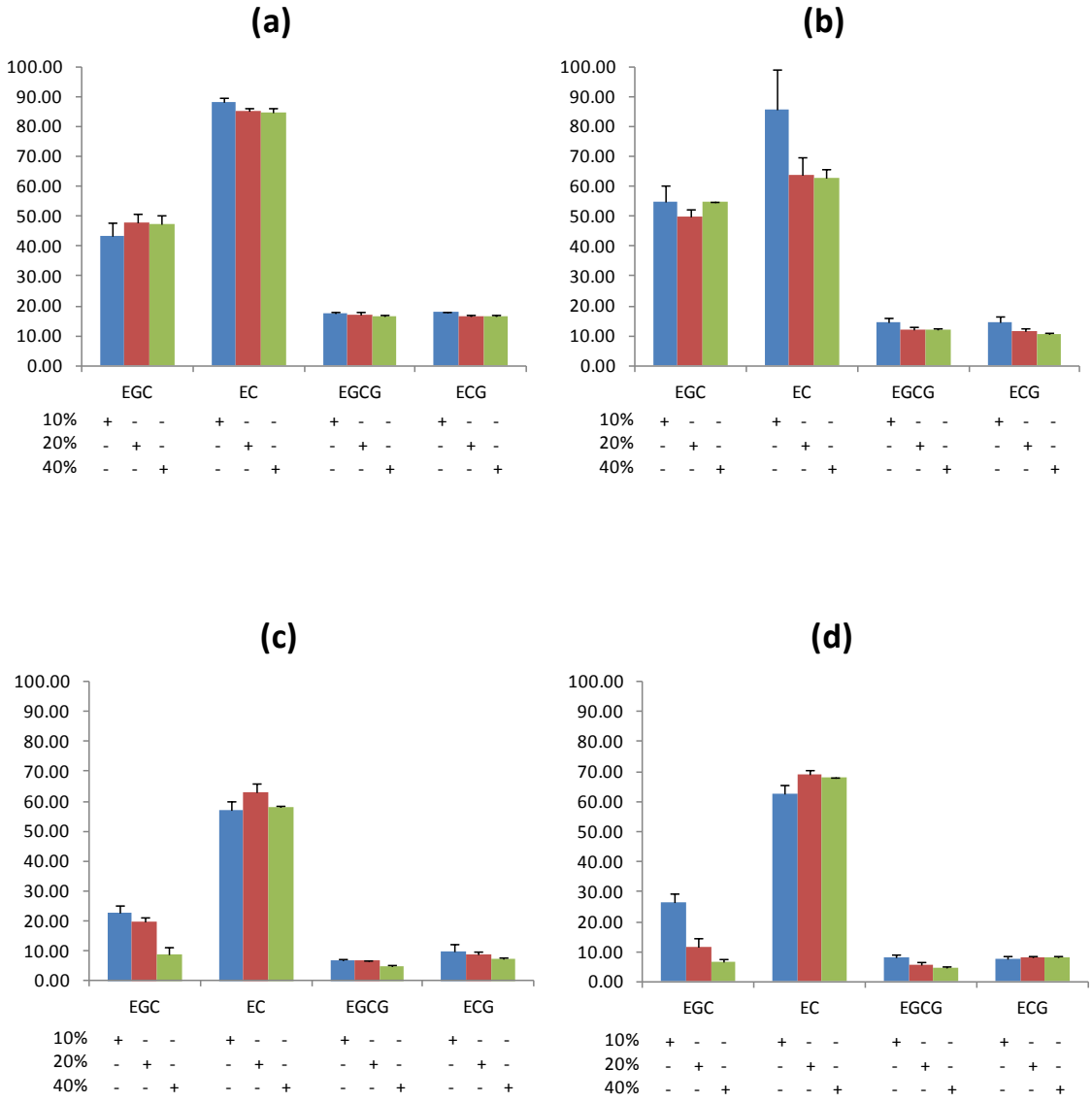

Figure A. 6: Relative bioaccessibility (%) of EGC, EC, EGCG, and ECG from green tea beverages formulated with (a) α-lactalbumin, (b) β-lactoglobulin, and (c) sodium caseinate in buffer, and (d) sodium caseinate in water.

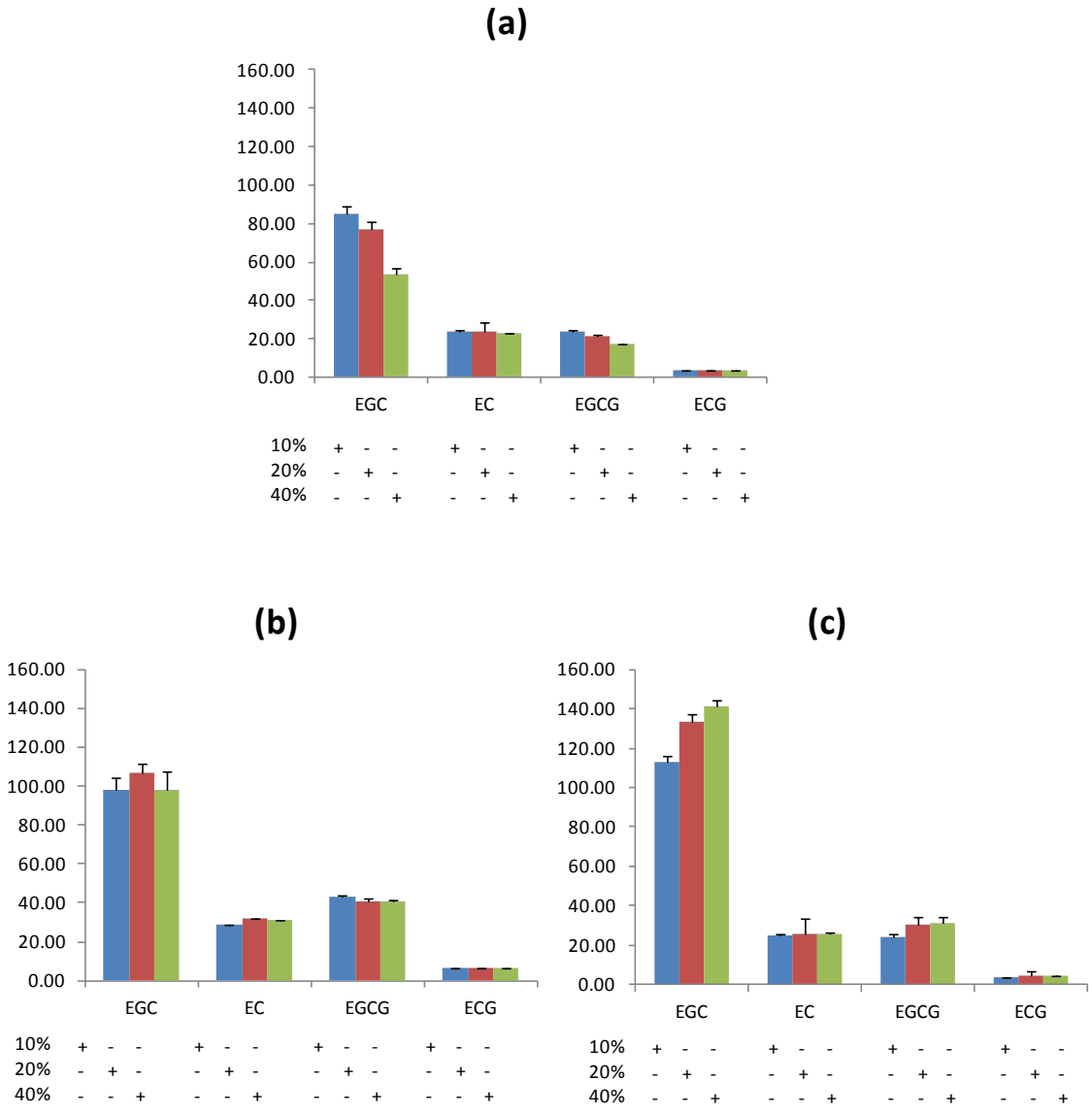

Figure A. 7: Absolute bioaccessibility (μg/g) of EGC, EC, EGCG, and ECG from green tea beverages formulated with (a) water, (b) buffer, and (c) nonfat dry milk

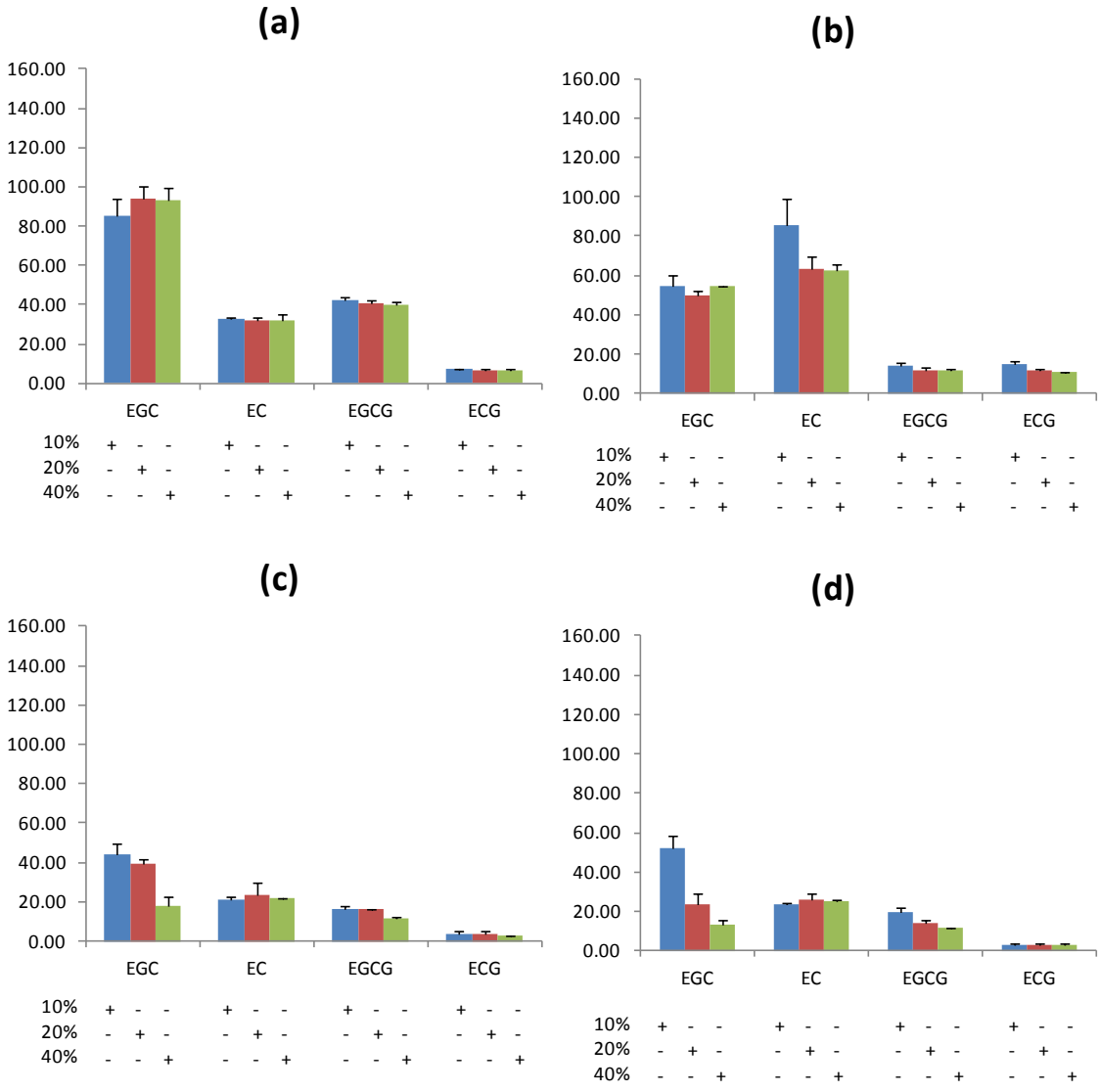

Figure A. 8: Absolute bioaccessibility (μg/g) of EGC, EC, EGCG, and ECG from green tea beverages formulated with (a) α-lactalbumin, (b) β-lactoglobulin, and (c) sodium caseinate in buffer, and (d) sodium caseinate in water.

Appendix B: Protein Profiles of Raw Materials used for Digestions Preceding SDS-PAGE and Fluorometric Analysis

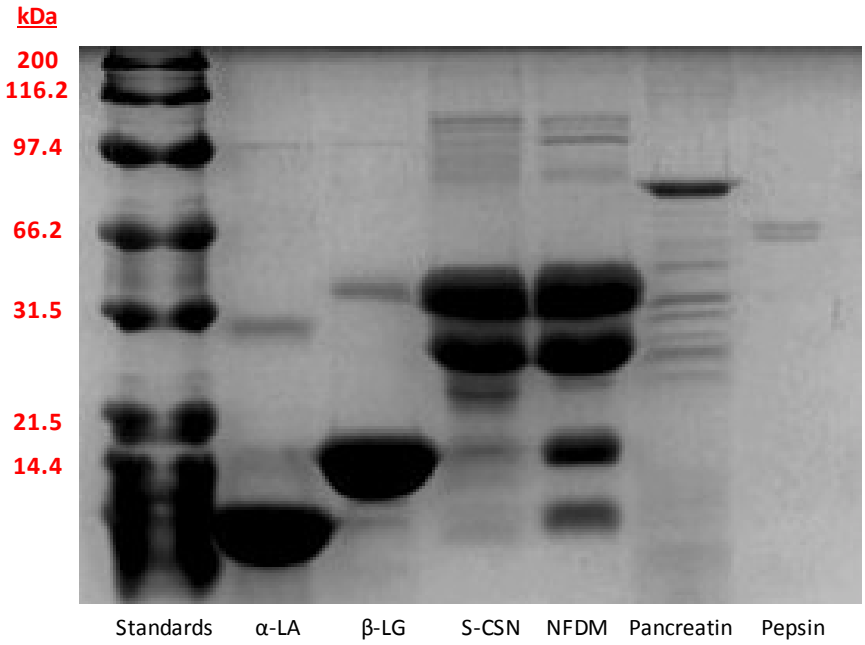

Figure B.2: Protein profiles of milk proteins and digestive enzymes used for digestions preceding SDS-PAGE and fluorometric analysis.

Appendix C: Fluorescence Quenching of Trp Residues by EGCG

The data shown in appendix B is select preliminary data (Table C.1 and C.2) and data included in section 3.7 (Table C.3 and C.4), presented in table format. Method development was initiated following a method by Kanakis et al. 2011. The fluorescence spectra were obtained using λ_{exc}=280 nm and λ_{emm} from 290-500 nm and the intensity at 350 nm (tryptophan (Trp)) was recorded.

Preliminary tests showed that EGCG mixed 1:1 with Tris-HCl (buffer) fluoresced less than just Tris-HCl (Table C.1). In addition, it appeared EGCG quenched digestive enzyme fluorescence. Due to its low impact on fluorescence and ability to quench some enzyme fluorescence, EGCG flurescence was not corrected for in calculations for percent quenching of Trp residues by EGCG.

Table C. 3: Fluorescence Intensity of EGCG and Digestive Enzyme Cocktail

Sample	**Average Fluorescence**
Tris-HCl	135.1“1.60
Tris-HCl + EGCG	97.1“1.05
Tris-HCl + Enzymes	404.1“2.06
Enzymes + EGCG	307.1“5.01

Fluorometric experiments were carried out on a Perkin-Elmer LS55 Spectrometer (Woodbridge, ON, Canada). Method development was initiated following a method by Kanakis et al. 2011. The fluorescence spectra were obtained using λ_{exc}=280 nm and λ_{emm} from 290-500 nm and the intensity at 350nm (tryptophan (Trp)) was recorded. Preliminary fluorometry results showed that both β-LG and enzyme cocktails elicit very high fluorescent responses. After several tests, it was determined that responses of β-LG solutions with concentration between 0.2-0.4 μM were within instrument’s linear range of fluorescence. Literature on protein-polyphenol binding report protein:polyphenol ratios (P:P) of 10:1 down to 1:27 (Hasni et a. 2011, Kanakis et al. 2011, Soares et al. 2007). Ratios ranging from 10:1 to 1:2 protein:polyphenol were tested keeping protein

concentration constant (0.3 µM) and using EGCG as the model polyphenol, however the noise was bigger than the change in response, making it difficult to guage the relative extent of protein-polyphenol binding. Therefore, ratios ranging from 1:2 to 1:40 protein:polyphenol were assayed, again keeping protein concentration constant (0.3 µM). For each subsequent decrease in milk protein:EGCG (MP:EGCG) at these levels, fluorescence decreased, indicating that mixtures containing the aforementioned MP:EGCG can be utilized to demonstrate the ability of EGCG to quench protein fluorescence. The objective of this assessment is to determine binding of EGCG to native and digested milk protein. Pepsin and pancreatin both contain Trp residues, thus the amount of fluorescence they contribute to total protein fluorescence in digesta was a concern. Therefore, 10-fold dilutions of enzyme cocktails containing pepsin and pancreatin were assayed, starting at concentrations used for *in vitro* digestion of green tea extract beverages (1.0 and 1.2 mg/mL, respectively), with and without β-LG. The fluorescent response of cocktails containing 0.01 and 0.012 mg/mL pepsin and pancreatin, respectively, was low in comparison to that of β-LG. The enzyme substrate ratio (E:S) used for *in vitro* digestion of green tea extract beverages in this study was about 2:1. Final digesta containing 0.5 µM milk protein and 0.01 mg/mL pepsin and 0.012 mg/mL pancreatin, for E:S ratio of 2:1 was used to obtain a clear fluorometric response from milk protein with little confounding fluorescence from digestive enzyme. According to literature, this E:S is sufficient for the digestion of B-LG (Mandalari et al. 2009, Otte et al. 1997). Furthermore, a 12 µM solution of EGCG was prepared to be mixed 1:1 with digesta for final MP:EGCG of 1:24 to force interactions between milk protein/peptides

and EGCG so that hypotheses concerning the effect of protein digestion on protein-polyphenol interactions can be made.

An EGCG stock solution (12 µM in Tris-HCl (10 mM, pH 7.4)) was prepared and kept on ice for analysis. EGCG was stable under these conditions with 98% recovery over 6 hr. S-CSN and β-LG digested to various extents *in vitro* were mixed 1:1 with EGCG by inverting solution 10 times. EGCG and protein concentrations of each solution were about 6 and 0.25 µM, respectively. Pepsin and pancreatin were also present from background digestion enzyme mixes at 0.005 and 0.006 mg/mL, respectively, therefore, blanks containing milk protein and background digestive enzyme mix mixed 1:1 with Tris-HCl (pH 7.4) replacing EGCG (12 µM) were prepared. The fluorescence spectra were obtained using λ_{exc}=280 nm and λ_{emi} from 290-500 nm. The intensity at 350 nm (Trp) was recorded.

The percentage fluorescence quenched was calculated using the following formula:

$$\%F_q=[(F_0-F)/F_0]100$$

Where: F_0= initial fluorescence intensity (intensity of blank)

F= fluorescence intensity in the presence of the quenching agent (intensity of protein-EGCG solution)

The i*n vitro* digestion procedure used prior to analysis by SDS-PAGE (outlined in section (2.6.1) was used with minor modification to digest S-CSN and β-LG in

preparation forfluorescence analysis. S-CSN and β-LG solutions (0.023 g/L and 0.035 g/L) were prepared in Tris-HCl (10 mM, adjusted with NaOH to pH 7.4). Final digesta were normalized to half starting protein concentration, 0.005 mg/mL pepsin, and 0.006 mg/mL pancreatin and had final pH of 7.4. Gastric time zero (G0) samples were prepared by addition of 1mL pepsin solution (0.07 mg/mL in 0.1M HCl) and 1 mL pancreatin solution (0.084 mg/mL in 0.1 M $NaHCO_3$) to 7 mL milk protein solution. G0 samples were made up to 14 mL with Tris-HCl, pH adjusted to 7.4, nitrogen gas and frozen (-20°C). For remaining samples, protein solutions (7mL) were pH adjusted to 3.0±0.1 (by addition of 1 M HCl) and made up to 8 mL with saline. The gastric phase was initiated by addition of 1mL pepsin solution (0.07 mg/mL in 0.1 M HCl). Pancreatin (1 mL, 0.084 mg/mL in 0.1 M $NaHCO_3$) was added to gastric time zero (G0) samples which were then made up to 14 mL with Tris-HCl, pH adjusted to 7.4, and frozen (-20°C). The duodenal phase was initiated in remaining solutions by pH adjustment to 6.5±0.1 and addition of 1mL pancreatin (0.084 mg/mL in 0.1 M $NaHCO_3$). Samples representing completed gastric digestion (G1) were made up to 14 mL with Tris-HCl, pH adjusted to 7.4, and frozen (-20°C). The pH of remaining samples was readjusted to pH 6.5±0.1. The solutions were made up to 12 mL with saline, blanketed with nitrogen gas and incubated in a shaking water bath (85 opm, 37°C). Digestions were stopped at either 15, 30, 60, or 120 mins (D15, D30, D60, D120), made up to 14 mL with Tris-HCl, pH adjusted to 7.4, and frozen (-20°C).

The ability of select milk proteins to alter digestive stability and by extension bioaccessibility of flavan-3-ols led us to further investigate the nature of EGCG binding to intact protein and peptides resulting from various extents of digestion by fluorescence

spectroscopy (Figure 11). EGCG was selected based on its abundance in tea and the high likelihood of its association with individual milk proteins (Poncet- Legrand 2007; Hasni et al. 2011; Baxter et al. 1997; Xiao et al. 2011; Xu & Chen 2011). This method assessed the amount of Trp fluorescence quenched ($\%F_q$) in individual protein by EGCG titration. Proteins (β-LG and S-CSN) were prepared in Tris-HCl, digested to various extents by pepsin and pancreatin at pH 3 and 6.5, respectively. The digesta were pH adjusted to 7.4 before analysis by fluorometry and mixed with EGCG solutions (pH 7.4). Final solutions contained ~0.25 μM protein and ~6 μM EGCG. These levels were chosen based on preliminary experiments that showed favorable concentrations for interactions between milk protein/peptides and EGCG so that hypotheses concerning the effect of protein digestion on protein-polyphenol interactions could be made without confounding precipitation.

$\%F_q$ by EGCG was greatest for both β-LG and S-CSN gastric digesta (G1), with $\%F_q$ of 13.23±0.90 and 12.39±1.06%, respectively (Figure 11). Furthermore, $\%F_q$ of β-LG G1 by EGCG was significantly greater ($p<0.05$) than quenching of undigested protein (6.67±0.65%) from each duodenal digesta. Specifically, $\%F_q$ of β-LG D15, D30, D60, and D120 were 6.86±1.02, 3.18±0.72, 4.78±0.75, and 5.83±1.85% respectively. However, $\%F_q$ of S-CSN G1 by EGCG was significantly greater ($p<0.05$) than only $\%F_q$ of S-CSN G0 (5.83±1.0%) and S-CSN D120 (5.12±1.07%). Interestingly, $\%F_q$ of undigested protein was not significantly different ($p>0.05$) for D15, D30, D60, and D120 digesta for both proteins. Additionally, EGCG quenched more fluorescence from S-CSN peptides resulting from 15, 30, and 60 minutes of duodenal digestions compared to β-LG peptides with 9.84±2.40, 5.79±0.22, and 8.15±1.01$\%F_q$ compared to 6.86±1.02,

3.18±0.72, and 4.78±0.75%F_q, however this trend is not significant. Although %F_q of undigested protein and duodenal digesta were low compared to G1 digesta, it appears that some binding was still occurring.

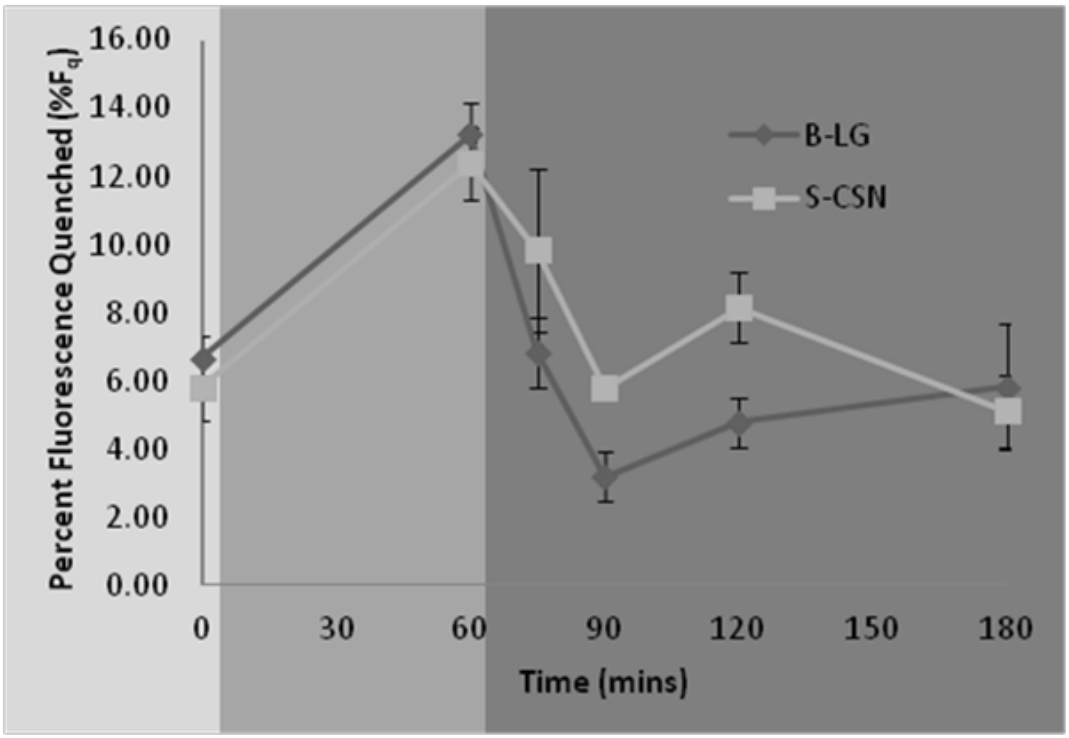

Figure C. 1: Percent Trp fluorescence quenched by EGCG of S-CSN digested to various extents. Digesta pH was adjusted to 7.4 and mixed 1:1 with Tris-HCl (pH 7.4) or EGCG (pH 7.4, 12 μM) for analysis by fluorescence spectroscopy. The lightest area of the graph corresponds with undigested protein, followed by protein digested gastrically, and protein digested duodenally.

Table C. 4: Percent Trp fluorescence quenched by EGCG of both β-LG and S-CSN digested to various extents.

(a)

Extent of Digestion	$\%F_q$
G0	6.67 ± 0.65
G1	13.23 ± 0.90
D15	6.86 ± 1.02
D30	3.19 ± 0.72
D60	4.78 ± 0.75
D120	5.83 ± 1.85

(b)

Extent of Digestion	$\%F_q$
G0	5.83 ± 1.00
G1	12.39 ± 1.06
D15	9.84 ± 2.40
D30	5.79 ± 0.22
D60	8.15 ± 1.02
D120	5.12 ± 1.07

Table C. 3: Trp fluorescence intensity of S-CSN solutions with and without EGCG. Digesta pH and EGCG (12 μM) or Tris HCl were mixed 1:1 maintaining pH of digesta for analysis by fluorescence spectroscopy. Each line is one of four different digestion procedures: (1) S-CSN-EGCG binding effects due to digestive pH (gastric pH 3.0, duodenal pH 6.5) (2) extent of binding between EGCG and digestive enzymes and (3) combined effect of protein digestion, pH, and presence of enzymes in S-CSN digesta on fluorescence quenching of protein Trp residues by EGCG. Superscript star denotes significant difference ($p<0.05$) between sample with and without quencher.

Average Fluorescence Intensity at 350nm			
Treatment	Extent of Digestion	- Quencher	+ Quencher
(1) S-CSN, no enzymes, pH 3 and 6.5	GpH0	393.01 ± 7.28	275.43 ± 1.47*
	GpH1	398.16 ± 1.66	268.50 ± 2.79*
	DpH15	451.73 ± 13.37	278.67 ± 24.97*
	DpH30	420.10 ± 6.65	247.61 ± 8.30*
	DpH60	429.60 ± 4.26	260.17 ± 7.97*
	DpH120	405.12 ± 6.75	239.69 ± 2.91*
(2) Enzymes, pH 3 and 6.5	EG0	225.16 ± 11.98	182.13 ± 10.93*
	EG1	222.77 ± 9.09	171.33 ± 6.35*
	ED15	317.85 ± 23.49	280.12 ± 20.02*
	ED30	298.61 ± 45.21	258.67 ± 40.62*
	ED60	251.78 ± 12.24	207.01 ± 5.73*
	ED120	243.32 ± 7.85	202.17 ± 6.29*
(3) S-CSN, enzymes, pH 3 and 6.5	FG0	463.12 ± 11.01	402.56 ± 13.35*
	FG1	449.33 ± 5.36	374.37 ± 6.04*
	FD15	598.30 ± 7.56	507.44 ± 11.33*
	FD30	579.85 ± 6.21	499.61 ± 9.06*
	FD60	559.94 ± 6.63	482.08 ± 10.46*
	FD120	575.11 ± 8.78	500.24 ± 8.96*

Table C.4: Percent Trp fluorescence quenched of S-CSN solutions by EGCG. Protein solutions and EGCG (12 μM) or Tris HCl were mixed 1:1 for analysis by fluorescence spectroscopy. Each line is one of four different procedures used to determine: (1) extent of binding between EGCG and digestive enzymes (2) S-CSN-EGCG binding effects due to digestive pH (gastric pH 3.0, duodenal pH 6.5) and (3) combined effect of protein digestion, pH, and presence of enzymes in S-CSN digesta on fluorescence quenching of protein Trp residues by EGCG. Different letters between treatments within a specific extent of digestion denotes significant difference ($p<0.05$).

Extent of Digestion	**Treatment**	**%F_q**
G0	1	19.18 ± 1.10[b]
	2	29.83 ± 1.63[a]
	3	13.06 ± 2.36[a]
G1	1	23.01 ± 1.43[b]
	2	32.57 ± 0.48[a]
	3	16.69 ± 0.49[a]
D15	1	14.76 ± 1.57[c]
	2	42.61 ± 3.92[b]
	3	15.18 ± 1.60[a]
D30	1	13.56 ± 0.43[c]
	2	41.09 ± 1.39[b]
	3	13.85 ± 0.88[a]
D60	1	16.02 ± 0.84[a]
	2	39.45 ± 1.65[b]
	3	13.91 ± 1.41[a]
D120	1	16.96 ± 0.56[a]
	2	40.77 ± 1.50[b]
	3	14.45 ± 2.23[a]

Appendix D: Acid Casein Technical Data Sheet

Technical Data Sheet	Issue Date: 11 May 2009	Approved By: _____
Kerrynor™ A290 Acid Casein		Page 1 of 2

PRODUCT DESCRIPTION
High protein milk extracts prepared by the acid precipitation of fresh skimmed milk.

TYPICAL CHEMICAL COMPOSITION

Fat %	1
Saturated %	0.63
Trans %	0.01
Protein % (as is)	86
Moisture %	10
Mineral Content %	2
Lactose %	0.1
pH	4.5

TYPICAL NUTRITIONAL DATA (per 100g)
Energy 353 kcal / 1500 kJ

FLAVOUR
Clean- Bland.

COLOUR
White to creamy in colour.

TEXTURE
A uniform, consistent powder.

TYPICAL MICROBIOLOGICAL DATA

T.B.C.	10,000/g
Coliform	Absent/0.1g
Yeast & Mould	<50 / <50/g
Salmonella	Absent/25g

MINERAL PROFILE

Calcium %	0.06
Sodium %	0.06
Potassium %	0.13
Phosphorus %	0.70
Magnesium %	0.01
Chloride %	0.15

CLAIMS
Ingredients are free from genetic modification.

SHELF LIFE & STORAGE CONDITIONS
A minimum of 24 months when stored in a cool dry, odour free store, at a maximum humidity of 65%.

AMINO ACID PROFILE (g/100g of amino acids)

Alanine	2.82
Arginine	3.44
Aspartic Acid	6.60
Cystine	0.33
Glutamic Acid	20.26
Glycine	1.81
Histidine	2.68
Isoleucine	4.97
Leucine	8.75
Lysine	7.46
Methionine	2.56
Phenylalanine	4.86
Proline	10.63
Serine	5.54
Threonine	4.28
Tryptophan	1.48
Tyrosine	5.34
Valine	6.19

TYPICAL PARTICLE SIZE
90 Mesh.

PACKAGING
Multi-walled paper sack with inner polyethylene liner.

CODING
Production code, Product description, Unit number and Unit weight.

Nutritional Analysis per 100.00 grams

Total Calories	353.00
Calories from Total fat	9.00
Total Fat (g)	1.00
Saturated (g)	0.63
Trans (g)	0.01
Cholesterol (mg)	<* 3.00
Sodium (mg)	60.00
Total carbohydrate (g)	0.10
Sugars (g)	0.10
Dietary Fiber (g)	0.00
Protein (g)	86.00
Vitamin A (iu)	35.00
Vitamin C (mg)	0.40
Calcium (mg)	60.00
Iron (mg)	0.55
Folic Acid (mg)	0.00

- *< Indicates less than value shown.

Figure D. 2 Technical Data Sheet for Acid Precipitated Casein

Appendix E: Specific Methodology

Preparation of sodium caseinate

Stock Solutions
Acid precipitated casein
Tris-HCl (10 mM, pH 7.4)
1M NaOH

Procedure:
1. Prepare acid precipitated casein in Tris HCl
2. Adjust pH to 6.3 using 1M NaOH solution
3. Stir consistently for 2 hr using magnetic stir bar maintaining pH at 6.3 until powder is completely dissolved
4. Store at 4°C for up to a week

In Vitro Digestion for Subsequent SDS-PAGE Analysis of Digesta

Stock Solutions
20 mg/mL pepsin in 0.1 M HCl
30 mg/mL pancreatin
0.9% saline

Procedure:

Gastric Phase
1. Place 14 mL tubes containing beverage (5.6 mL) on ice.
2. Bring up to about 8.4 mL by addition of 0.9% saline.
3. Adjust pH to 3.0 ± 0.1 using 1.0 M HCl. Record volume and pH.
4. Add 0.56 mL of pepsin solution to each tube.
5. Bring up to about 11 mL by addition of 0.9% saline.
6. Collect and freeze (-20°C) samples representing gastric time zero.
7. Blanket remaining samples with nitrogen gas, cap, parafilm.
8. Incubate in shaking water bath at (37°C, 85 opm,1 hr)

Intestinal Phase
1. Remove tubes from water bath and place on ice.
2. Freeze gastric digests representing 1 hour gastric digestion (-80°C) and freeze dry.
3. Add 1.0 M $NaHCO_3$ to raise pH to 6.5 ± 0.1. Record volume and pH.
4. Add pancreatin solution (0.56 mL) to each tube.
5. Check pH and readjust if needed.
6. Bring up to 14 mL by addition of 0.9% saline.
7. Blanket with nitrogen gas, cap, and parafilm.
8. Incubate in shaking water bath at (37°C, 85 opm, ≤2 hr).

9. Freeze samples representing various extents of duodenal digestion (-80°C) and freeze dry.

SDS-PAGE analysis of sample digests

Stock Solutions:
Polyacrylamide gel
Tris-HCl (10 mM, pH 7.4)
Bio-Rad 2x Laemmli buffer
2% Coommassie Blue solution
Destaining solution (30% methanol, 10% acetic acid, 60% dd H_2O
Molecular weight marker calibration kit

Procedure:
1. Resolubilize freeze-dried digest in Tris-HCl for about 8 mg/mL total test protein.
2. Mix samples 1:1 with Bio-Rad 2x Laemmli buffer for 42 µL total solution and boil (5 minutes).
3. Load samples (40 µL) to gel and molecular weight ladder (10 µL) onto gel.
4. Run gel at 100V for about 1.5 hours.
5. Stain gel with Coomassie Blue solution (30 min).
6. Destain gel for 10 minutes in destain solution. Dispose of destain, and repeat 10 minute destain a few times. Continue to destain overnight or until gel background is clear.

In Vitro Digestion for Subsequent Fluorometric Analysis of Digesta

Stock Solutions
0.07 mg/mL pepsin in 0.1M HCl
0.084 mg/mL pancreatin
0.9% saline
Tris-HCl (10 mM, pH 6.5 and 3.0)

Procedure:

Gastric Phase
1. Place 14 mL tubes containing beverage (7.0 mL) on ice.
2. For gastric time 0 samples (G0):
    a. add pepsin solution (1 mL) and pancreatin solution (1 mL), bring up to 14 mL total with Tris-HCl (pH 3.0), pH adjust to 3.0, and freeze (-20°C).
3. For remaining samples:
    a. Adjust pH to 3.0 ± 0.1 using 1.0 M HCl (record volume and pH) .
    b. Bring beverage up to about 8 mL by addition of 0.9% saline.
    c. Add 1 mL of the pepsin solution to each tube.

d. Blanket with nitrogen gas, cap, parafilm.
e. Incubate in shaking water bath at (37°C, 85 opm, 1 hr)

Intestinal Phase

4. Remove tubes from water bath, place on ice
5. For gastric time 1 hr samples (G1):
    a. Add pancreatin solution (1 mL), bring up to 14 mL total with Tris-HCl (pH 3.0), pH adjust to 3.0, and freeze (-20°C).
6. For remaining samples:
    a. Add 1.0 M $NaHCO_3$ to raise pH to 6.5 ± 0.1 (record volume and pH).
    b. Add pancreatin solution (1 mL) to each tube.
    c. Check pH, and readjust if needed.
    d. Bring up to 12 mL by addition of 0.9% saline.
    e. Blanket with nitrogen gas, cap, and parafilm.
    f. Incubate in shaking water bath at (37°C, 85 opm,≤2 hr).
    g. Bring samples representing various extents of duodenal digestion to 14 mL with Tris-HCl pH 6.5, pH adjust to 6.5 if needed, and freeze (-20°C)

Fluorometric Analysis

Instrument:	Perkin-Elmer LS55 Spectrometer
Excitation wavelength:	280nm
Emmission wavelength:	290-500
Wavelength of interest (Trp):	350nm

Procedure:

1. Pipette sample and with either Tris-HCl or quencher into cuvette 1:1
2. Invert solution ten times
3. Wipe down cuvette with Kim Wipe
4. Place in fluorometer
5. Record fluorescence